CONTROL OF MAP-2 INTERACTIONS WITH MICROTUBULES BY ENZYMATIC PHOSPHORYLATION

By

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This dissertation is dedicated to my family
for their endless support and encouragement over the years.

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ABBREVIATIONS

ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	3′,5′-cyclic adenosine monophosphate
cDNA	complimentary DNA
CNBr	cyanogen bromide
dNTP	deoxy-nucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)
GTP	guanosine triphosphate
IPTG	isopropyl β-D-thiogalactopyranoside
MAP	microtubule-associated protein
Mes	2-[N-morpholino]ethanesulfonic acid
Mops	3-[N-morpholino]propanesulfonic acid
M_r	relative molecular weight
MT	microtubule
MTBR	microtubule-binding region
mRNA	messenger RNA
PCR	polymerase chain reaction
SDS	sodium dodecyl sulfate

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Chairperson: Daniel L. Purich
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Microtubules and their associated proteins (MAPs) regulate various cellular processes including shape, vesicular transport and cellular reorganization. Phosphorylation of these microtubule-associated structural proteins represents one mechanism for controlling these functions. MAP-2, a family of three neuronal proteins, is extensively phosphorylated on serine and threonine residues with 46 phosphoryls found covalently bound to the adult isoforms *in vivo*. Although MAP-2 phosphorylation has been studied *in vitro* and *in vivo*, the exact location of phosphorylated sites that influence the strength of MAP-2 binding to microtubules (MTs) has remained elusive. A novel strategy for localization is discussed throughout this dissertation.

MAP-2 consists of at least two distinct domains: an M_r 240 kD projection arm and an M_r 28 kD MT-binding region (MTBR). The greatest extent of phosphorylation is evident in the projection arm; however, the MTBR retains nearly all the binding affinity of intact MAP-2 for MTs.

This smaller region contains three non-identical repeat sequences designated m_1, m_2, and m_3 with the second one contributing significantly to the binding energy of MAP-2. I initially evaluated the effects of phosphorylation by various protein kinases (PKA, PKC and proline-directed kinase) and observed that PKC altered MAP-2 interactions with MTs most dramatically. Accordingly I focused my efforts on understanding the phosphorylation affects of MTBR binding to MTs and promoting microtubule assembly using protein kinase C as an *in vitro* probe. Bacterially expressed MAP-2 MTBR, containing newly introduced CNBr-cleavable methionyl residues, as well as mass spectrometry and site-directed mutagenesis were used to locate and confirm assignments of critical phosphorylation sites.

This dissertation addresses in part the localization and role of phosphoryls on two specific residues (Ser-1703 and Ser-1711) in terms of kinase-mediated control of MTBR-stimulated tubulin polymerization. Ser-1703 is located between m_1 and m_2, while Ser-1711 is within the second non-identical repeat. Upon more extensive PKC action, MT binding is abolished completely by phosphorylation of Ser-1728 (located between the 2nd and 3rd repeat). This effect, however, is conditionally dependent on phosphorylation at Ser-1703 and/or Ser-1711. My results suggest that *in vivo* binding of MAP-2 to microtubules may be controlled by protein kinase action at these residues. Additionally, the strategy presented here may simplify future *in vivo* studies.

CHAPTER 1
INTRODUCTION

The Cytoskeleton: An Overview

The cytoskeleton is a versatile and complex eukaryotic organelle whose structural and dynamic properties are essential for maintaining a variety of cellular functions. The cytoskeleton maintains cell shape, while still permitting cell movement, and it is also involved in vesicular trafficking, chromosome movement, and anchoring of membrane-bound transporters. Three types of fibers and their associated proteins establish this internal architecture of the cytoplasm: microfilaments, intermediate filaments, and microtubules. The integral nature of these structures and the high degree of evolutionary conservation among species indicates the importance of the cytoskeleton in sustaining cellular dynamics.

Microfilaments, each 7 nm in diameter, are composed of actin monomers which assemble in an ATP-dependent manner (Pollard, 1990). Electron micrographs and immunofluorescent studies reveal filamentous actin has a two chain helical structure in which the head-to tail self-assembly mechanism leads to intrinsic structural polarity. Treadmilling occurs between the fast and slow growing ends *in vitro* and *in vivo*. Eukaryotic cells have six actin isoforms: two in striated muscle, two in smooth muscle and two in non-muscle cells. Although three isoelectric variants, referred to as α-, β- and γ actin, have been isolated, their apparent polymerization properties are indistinguishable (Whalen *et al.*, 1976). In non-muscle cells, actin filaments line the periphery of the cell to propagate cell motility and this localization facilitates rapid responses to chemotaxis components.

Unlike actin, intermediate filaments (IFs) are divergent structures that are cell type-specific and are generated from a variety of protein subunits (Steinert and Roop, 1988). The various IF proteins differ considerably in molecular weight and isoelectric charge, but all share a common conserved molecular arrangement from the amino to carboxyl termini: head, rod and tail domains. In neurons, neurofilament (NF) triplet proteins (comprised of high, medium and low molecular weight polypeptide chains) are extensively phosphorylated in their C-terminal tail domains. Both NF-M and NF-H co-assemble with NF-L, and the latter acts as a scaffold; indeed, in the absence of NF-L, neurofilaments cannot form. Neurofilaments, which appear in electron micrographs with 10 nm diameters, are thought to play a role in establishing and maintaining axonal caliber and consequently, cellular insulation (Hoffman *et al.*, 1987). No other activities are currently known for these stable proteins.

Microtubules (MT) are the largest (25 nm in diameter) of the cytoskeletal components. MTs are composed of an α- and β-tubulin heterodimer with GTP initially bound on both subunits. *In vitro* studies reveal that upon nucleotide hydrolysis (GTP → GDP) on the exchangeable site of β-tubulin, a three start helical structure nucleates to form the polymerized filament; the α-subunit has a non-exchangeable GTP binding site (Purich and Kristofferson, 1984). Similar to microfilaments, microtubules display structural polarity with a fast and slow growing end, and they display dynamic instability *in vitro* and *in vivo* with a loss of the GTP cap at both its ends (Mitchison and Kirschner, 1984). The GTP cap denotes a boundary layer of GTP-bound tubulin which stabilize the MT ends. Recently γ-tubulin was identified as a new subpopulation in the tubulin superfamily (Oakley and Oakley, 1989). Antibodies against this protein do not stain microtubules; rather, they reveal spindle pole bodies (Oakley *et al.*, 1990) Investigators suggest that γ-tubulin may nucleate αβ composed MTs at the centrosome (Joshi *et al.* 1992), the so-called microtubule-organizing center (MTOC). Any direct interactions, however, remain to be elucidated.

In addition to actin, intermediate filament proteins, and tubulin, the cytoskeleton often contains other less abundant associated proteins. Filaments may interact with other organelles and with each other through such associated macromolecules. In terms of actin polymerization there are numerous families of molecules which include both monomeric sequestering proteins (*e.g.*, profilin and actobindin) and filament capping and severing proteins (*e.g.*, gelsolin and villin). Microtubule-associated proteins (MAPs) are thought to influence cell dynamics *via* microtubule binding. MAPs have either structural (*e.g.*, MAP-2 and tau) or enzymatic activities (*e.g.*, kinesin and MAP-1c).

Enzyme-catalyzed covalent modification of these proteins can disrupt the dynamics of this organellar network. The remainder of the discussion in this chapter focuses on the roles of microtubules and their associated proteins individually as found during interphase. The neuronal cytoskeleton and MAP-2 will be featured. MAP-2 is known to be highly phosphorylated *in vivo*, and MAP-MT interactions are disrupted by enzymatic phosphorylation.

Tubulin

Identification and general properties. Biochemical analyses of microtubules began in the mid-1960's with the identification of a colchicine binding protein with a M_r of 100,000 daltons (Borisy and Taylor, 1967; Wilson and Friedkin, 1967). Weisenberg *et al.* (1968) used radiolabeled colchicine, an anti-mitotic drug, to first purify tubulin from rat brain homogenates. They visualized the α- and β- subunits by polyacrylamide gel electrophoresis, but were unable to reassemble tubulin *in vitro* until 1972 as shown in electron micrographs (Weisenberg, 1972). In preliminary studies, partially purified tubulin was incubated under several conditions to induce polymerization, thereby providing evidence for the requirement of GTP, magnesium, and EGTA. Additionally, calcium was shown to inhibit microtubule assembly, a finding numerous investigations have since confirmed (Olmsted and Borisy, 1975; Rosenfeld *et al.*, 1976). Currently, microtubule protein (MTP) preparations use repeated cycles of warm-induced assembly and cold-induced disassembly in a glycerol containing buffer (Shelanski *et al.*, 1973; Karr *et al.*, 1979). In conjunction with electron microscopy, microtubule assembly and disassembly properties are routinely measured as turbidity measurements in a spectrophotometer (Gaskin *et al.*, 1974). The two-step polymerization process has an initial lag phase for nucleation to occur, followed by a period of growth or elongation

(Bryan, 1976; Karr *et al.*, 1979; Barton and Riazi, 1980). The kinetics of both stages is tubulin concentration-dependent, as shown by dilution studies where critical concentrations of 0.2 mg/ml were measured (Gaskin *et al.*, 1974; Karr *et al.*, 1979; Barton and Riazi, 1980)

Structural properties. In solution, αβ tubulin remains predominantly associated in its dimeric form, and each subunit has an approximate molecular weight of 50,000 daltons. Luduena *et al.* (1977) first demonstrated that tubulin is an αβ heterodimer by crosslinking solubilized chick brain tubulin *via* dimethyl-3,3'-(tetramethylenedioxy) dipropionimidate followed by non-denaturing gel electrophoresis. Circular dichroism studies revealed purified tubulin contains nearly 50 percent random coil, 20 percent alpha-helical structure and about 30 percent beta sheet (Ventilla *et al.*, 1972). A change in the optical signal, attributed to conformational changes, was observed at elevated incubation temperatures (40-50°C) but the data indicated that both nucleotides and colchicine protect tubulin from such heat inactivation and potential denaturation. Amino acid sequence determination reveals both subunits are rich with glutamic acid residues (Valenzuela *et al.*, 1981; Ponstingl *et al.*, 1981) sharing 36-42 percent homology (Little and Seehaus, 1988). Despite their similarities, in mammals there are multiple α- and β-tubulin genes, four and six, respectively, and greater than 20 variant isoforms have been identified (Silflow and Rosenbaum, 1981; Field *et al.*, 1984). Post-translational modifications are subunit specific in the C-terminus region, with α-tubulin undergoing acetylation (L'Hernault and Rosenbaum, 1985), glutamylation (Edde *et al.*, 1990) and tyrosinylation (Raybin and Flavin, 1977) while β-tubulin is phosphorylated (Gard and Kirschner, 1985). Table 1-1 shows the carboxyl terminal sequences where α- and β-tubulin are most divergent. The significance of these modifications is still unknown, but it has been suggested that microtubules are rendered more or less stable by a particular modification. Indeed, Detrich *et al.* (1987) has shown cold-stable cod-fish microtubules are enriched with acetylated and tyrosinylated tubulin, although stability has not been directly demonstrated with these covalent modifications.

Nucleotide binding sites. Weisenberg *et al.* (1968) and Berry and Shelanski (1972) provided early evidence for the existence of two non-identical guanine nucleotide binding sites on each tubulin dimer. These sites were distinguished and characterized by their ability to exchange radiolabeled GTP from the medium. The exchangeable site (E-site) readily replaces GTP for GDP within 15 minutes, whereas the non-exchangeable site (N-site) does not exchange, even over several hours. These investigators proposed a head-to-tail polymerization model where one GTP binding site is buried, such that it cannot exchange as readily as the other. Use of 8-azido-GTP as a photoaffinity probe indicated that hydrolysis occurred on β-tubulin concurrently with polymerization (Geahlen and Haley, 1977; 1979). Jacobs *et al.* (1974) reported that ATP participates in transphosphorylation of GDP bound tubulin *via* nucleoside diphosphate kinase (NDPK). Additionally, David-Pfeuty *et al.* (1977) showed the participation of a GTPase activity in microtubule polymerization, and MacNeal and Purich (1978) showed that the exchangeable site GTP is hydrolyzed in a one-to-one stoichiometry relative to tubulin incorporation into MTs. Interestingly, Weisenberg

Table 1-1

Sequences and properties of vertebrate tubulin genes

Gene [a]	C-terminal sequence	Expression
Alpha subunit		
1	D-Y-E-E-V-G-V-D-S-V-E-G-E-G-E-E-E-G-E-E-Y	neuronal/ developmental
2	D-Y-E-E-V-G-V-D-S-V-E-G-E-G-E-E-E-G-E-E-Y	neronal/ constitutive
3	D-Y-E-E-V-G-G-V-S-V-E-A-E-E-A-E-E-G-E-E-Y	many tissues/ minor
4	D-Y-E-V-G-I-D-S-Y-E-D-E-D-E-G-E-E	testes/ major
Beta subunit		
1	E-E-E-E-D-F-G-E-E-A-E-E-E-A	many tissues/ constitutive
2	D-E-Q-G-E-F-E-E-E-G-E-E-D-E-A	neuronal/ major
3	E-E-E-G-E-M-Y-E-D-D-E-E-E-S-E-Q-G-A-K	neuronal/ minor
4a	E-E-G-E-F-E-E-E-A-E-E-E-V-A	brain specific/ major
4b	E-E-E-G-E-F-E-E-E-A-E-E-E-A-E	testes and other/ major
5	N-D-G-E-E-E-A-F-E-D-D-E-E-E-I-N-E	not in brain/ minor
6	D-V-E-E-Y-E-E-A-E-A-S-P-E-K-E-T	platelets/ major

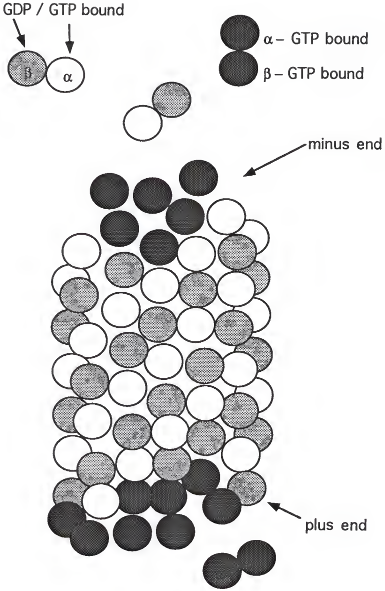
[a] All sequences correspond to mouse cDNA clones (Lewis *et al.*, 1985; Villasante *et al.*, 1986).

et al. (1976) reported that ATP-driven MT assembly shows an increased lag phase thus indicating a weakened nucleation phase with no apparent alteration in the overall extent of polymerization as compared to GTP-stimulated assembly. The basis for this lag was later explained by Terry and Purich (1980) who demonstrated that NDPK only phosphorylates unbound nucleotide, and the delay relates to GDP release and GTP rebinding. Likewise, Karr *et al.* (1979) used non-hydrolyzable GTP analogs to demonstrate that GTP is involved early in nucleation, while GDP stabilizes microtubules during elongation. More recently Angelastro and Purich (1992) discovered deoxy-GTP in the N-site of nerve growth factor-treated PC12 pheochromocytoma cells as well as embryonic chick dorsal root ganglion neurons.

Microtubules

Structural properties. Microtubules are hollow cylinders composed of longitudinally arranged protofilament arrays of $\alpha\beta$ tubulin (see Fig. 1-1). They have a plus (fast growing) and minus (slow growing) end; and these can be differentiated by the movement of plus and minus end directed motor proteins (*i.e.*, kinesin and dynein). The detailed packing of the globular subunits has been investigated by diffraction analysis of negatively stained flagellar and brain microtubules (Erickson, 1974; Amos and Klug, 1974). The data, from both populations, revealed a 4-nm axial periodicity and 8 nm wall and shows a heterologous interaction between protofilaments. In axonemes a double ringlet is observed with two single filaments surrounded by nine outer doublet microtubules composed of an A and B tubule. In general *in vitro*

Fig. 1-1: Illustration of a dynamic microtubule filament. Microtubules are composed of α and β tubulin subunits in a linear array. Both the plus and minus ends are dynamic and have a GTP cap which can be subsequently lost over time. The α subunit has GTP bound at all times (non-exchangeable), while the β subunit has either GTP or GDP bound (exchangeable). Nucleotide hydrolysis at the β-subunit occurs upon polymer formation as indicated by the black *versus* shaded circles.


GDP / GTP bound
β
α
α – GTP bound
β – GTP bound
minus end
plus end

electron microscopy studies reveal microtubules form ribbons, sheets and rings (Matsumura and Hayashi, 1976; McEwen and Edelstein, 1980). These unusual structures are buffer- and pH-dependent, and whether they occur in nature remains unclear. Additionally, the highly acidic nature of microtubules results in their association with over 350 drugs (*e.g.*, taxol, colchicine and vinblastine).

Microtubule assembly. In terms of polymerization there are three individual phases: nucleation, elongation and length redistribution. Nucleation and elongation as discussed earlier can be monitored spectrophotometrically with a distinct separation between the initial rate and steady-state kinetic properties. Length redistribution refers to the continuous dynamic instability properties of the microtubule ends as described both *in vitro* (Mitchison and Kirschner, 1984; Kristofferson *et al.* 1986) and *in vivo* (Sammak and Borisy, 1988). Dynamic instability refers to the continuous flux of shrinking and growing microtubules populations which rarely interconvert. Some microtubules continue to grow while others are lost by depolymerization at the distal ends. These investigators using electron and direct fluorescent microscopy techniques demonstrated that the kinetics at each end is independent of the other as evidenced under *in vivo* conditions where minus ends are held at the MTOC and thus are not free to undergo any exchange. The sudden loss of the GTP cap followed by a rapid shortening of the microtubule is termed a "catastrophe." Subsequently, the filaments can be "rescued" by the addition of tubulin dimers or the association with fibrous MAPs which blocks further depolymerization (Simon and Salmon, 1990; Kowalski and Williams, 1993). Bergen and Borisy (1980) and

Carlier and Pantaloni (1981) independently measured the affinity constants for both ends of the microtubule using electron microscopy and spectrophotometric measurements, respectively. Their data agree for the plus and minus ends having dissociation constants of 17 s^{-1} and 7 s^{-1}, respectively. Several investigators have shown a direct correlation between GTP hydrolysis and microtubule polymerization (Karr *et al.*, 1979; O'Brien *et al.*, 1987; Stewart *et al.*, 1990; Davis *et al.*, 1993). Correspondingly, Karr *et al.* proposed a boundary model where the GTP cap comprises the outermost layer of the microtubule lattice rather than a deep cap model (Carlier and Pantaloni, 1981). Interestingly, analysis of the carboxyl terminus of each tubulin subunit, where post-translational modifications are prevalent, reveals a control of MT assembly from this region. This part of the tubulin molecule has a conserved GTP binding motif (G-X-X-X-X-G-K) as well as a proposed site for MAP-MT interactions, and the region may also regulate interactions with drugs such as colchicine (Avila *et al.*, 1987). Subtilisin treatment selectively removes 4 amino acids from the C-terminal domain and results in a loss of MAP binding and assembly regulation (Dingus *et al.*, 1991).

Microtubule-Associated Proteins

MAPs constitute a diverse class of proteins that have been classified on the basis of their binding or enzymatic modification of microtubules. Considering the diversity of microtubule function, it was logically anticipated that there exist numerous proteins to regulate temporal, spatial and metabolic control of these processes (see Table 1-

2). While initial studies using repeated cycles of assembly and disassembly identified several fibrous MAPs (Shelanski *et al.*, 1973), this method precluded the discovery of several other transiently associated proteins.

Motor-driven MAPs

Motor proteins recognize the asymmetry of microtubules and move along MTs as though they are tracks with a defined polarity. Gibbons and Rowe (1965) first noted the ATP-dependent movement of axonemal dynein on the outer arms of the doublet microtubules. Axonemal dynein is a two-headed, minus-end directed motor protein which propagates a sliding motion by forming crossbridges between A- and B-tubules of adjacent doublets (Gibbons, 1988). This motor is composed of a heavy chain, two to three intermediate chains and four light chain polypeptides. Cytoplasmic dynein is morphologically indistinguishable from axonemal dynein (Vallee *et al.*, 1988), although composed of nine similar subunits (Paschal *et al.*, 1987). Cytoplasmic dynein is widely distributed and plays a role in retrograde organelle transport with rates of 1.3-2.0 μm/sec (Paschal *et al.*, 1987). Recently, Pfarr *et al.* (1990) and Steuer *et al.* (1990) showed its associated with kinetochore microtubules of the mitotic spindle, and the data suggest a direct involvement in chromosomal separation.

The kinesin superfamily of proteins is perhaps the best characterized of the motor proteins. Structurally, each consists of three domains: a globular, two-headed catalytic region, a coil-coil polypeptide stalk, and a fan-like tail (Amos, 1987; Scholey *et al.*,

Table 1-2

Major classes of microtubule-associated proteins

Family	Protein	Mr (kD)	length (nm)	Properties
Fibrous MAPs	MAP-1 ab	350	100-255	thermolabile
	MAP-2 ab	200	185	thermostable; two distinct regions with PKA and calmodulin binding sites; adult isoform; dendritic
	MAP-2 c	70	50	embryonic; axonal
	tau	55-65	56.1	thermostable; multiple isoforms; forms PHF structures upon phosphorylation; axonal
	MAP-4	210	100	ubiquitous; MAP-2 like MTBR
Motors	Kinesin	110	70-120	plus-ended; ATPase; 2-headed
	Dynein	varies	50	minus-ended; ATPase; flagellar; 2 and 3-headed molecules
	MAP-1 c	300	50	cytoplasmic dynein; 2-headed
Others	Katanin	60, 81	unknown	ATPase severing protein
	Clip-170	170	unknown	links endocytic vesicles to MTs

1989). The head domain is conserved among the members of this family with 40 percent homology, whereas other regions are highly divergent. Kinesins are plus-end directed, ATP-dependent motors which were first isolated as an activity which moved microtubules along latex beads at 2-4 μm/sec (Vale *et al.*, 1985). Immunofluorescence microscopy experiments indicate that kinesin is associated with organelles but unlike dynein, can transport vesicles *in vitro* without added cellular factors (Pfister *et al.*, 1989).

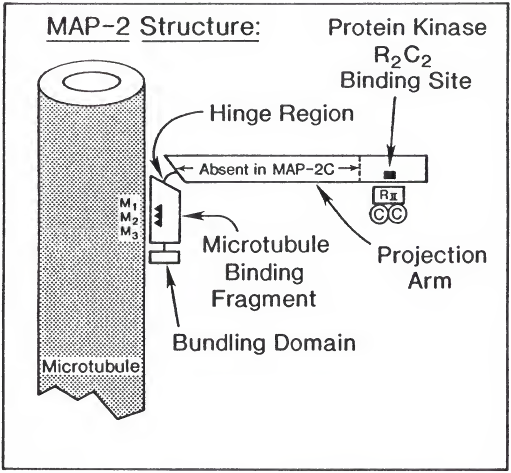
Cytoskeleton researchers have proposed that filaments are transported down axonal and dendritic processes. Two proposed models differ as to the location of microtubules assembly. Black and Lasek (1980) suggest polymeric forms are transported, whereas more recent studies by Weisenberg *et al.* (1987) suggest MTs are stationary with fewer constraints on the form of the transported protein. Okabe and Hirokawa (1990) injected fluorescein-labelled tubulin into cultured dorsal root ganglion cells and monitored tubulin incorporation into quiescent and growing neurites. Their data in conjunction with the work of Lim *et al.* (1989) strictly support the latter model with no evidence of tubulin polymers traversing the axon from the cell body. The motors involved in these processes, however, remain to be elucidated.

Fibrous MAPs

The use of brain extracts to assemble microtubules *in vitro* (Weisenberg, 1972) laid the foundation for the identification of cytoplasmic protein components that associate with the MT lattice. Fractions enriched in fibrous MAPs promoted MT assembly at lower critical concentrations than did tubulin alone (Weingarten et al., 1975; Sloboda et al., 1976). Among the best characterized of the non-motor MAPs are MAP-2, MAP-4, and tau proteins. Additionally, MAP-1a and -1b were identified in both axonal and dendritic processes as a major neuronal component (Bloom *et al.*, 1985). However, the inability to quantitatively purify this protein has hindered its biochemical analysis. Epitope mapping has revealed MAP-1a and -1b are structurally different from the other fibrous MAPs (Herrmann *et al.*, 1984) and have an unrelated microtubule-binding region (MTBR) (Lewis *et al.*, 1988).

Weingarten et al. (1975) were the first to purify tau proteins from porcine brain homogenates under non-stringent conditions. Herzog and Weber (1978) and Kim et al. (1979), however, heat-treated brain microtubule proteins as a means to isolate the thermostable proteins, MAP-2 and tau. Figure 1-2 shows the current working model of MAP-2 structure and binding to the microtubule lattice. MAP-2 is a multifunctional domain protein with two major regions: an acidic (pI 4.8) N-terminal projection arm domain that extends away from the microtubule; and a highly basic (pI 10.5) MT-binding domain (Flynn *et al.*, 1987). Earlier work by Vallee and Borisy (1977) demonstrated an proteolytic susceptibility of high-molecular-weight MAPs with selective removal of the projection arms *in vitro*. Interestingly, assembly properties of microtubule proteins were not altered by protease treatment when compared to untreated samples. This observation indicated that only the smaller fragment, now referred to as the MT-binding region, of MAP-2 was essential for MT polymerization. This has

Fig. 1-2: Schematic representation of MAP-2 binding to a fixed microtubule lattice. MAP-2 is a 200 kD multi-functional domain. Upon proteolytic treatment with thrombin MAP-2 is cleaved into a 190 kD projection arm and a 22 kD MT-binding region. The N-terminal, highly acidic (pI 4.8) projection arm contains a binding site for the regulatory subunit of protein kinase A and a splice site which is absent in the embryonic isoform. The C-terminal, highly basic (pI 10.5) binding region includes the three non-identical repeat motif.


MAP-2 Structure:
Protein Kinase
R_2C_2
Binding Site
Hinge Region
Absent in MAP-2C
R_{II}
C C
M_1
M_2
M_3
Microtubule
Binding
Fragment
Projection
Arm
Bundling Domain
Microtubule

led to the ardent investigations on the MT-binding region of fibrous MAP proteins which appear to interact with microtubules *via* a highly related motif.

Although the tissue and subcellular distribution of MAP-2, tau and MAP-4 is distinctly different (see Table 1-2), these proteins appear structurally related. Lewis *et al*. (1988) showed that MAP-2 and tau localize to the dendrite and the axon, respectively, and that they share non-identical tandem repeats of a sequence of 18 amino acid stretches, repeated three to four times (see Fig. 1-3). Upon further analysis, these repeats proved to be highly conserved in the MT-binding region of these proteins as well as in MAP-4. Independent reports by Joly *et al*. (1989) and Ennulat *et al*. (1989) revealed that synthetic peptides corresponding to the second repeated sequence in either MAP-2 or tau, respectively, competed with native protein and promoted MT assembly. Additionally in tau, the first repeat was also shown to bind to microtubules; this illustrated that MAP-2 and tau, although structurally quite similar, are distinct proteins and this may account for their specific roles in dendrites and axons. Doll *et al*. (1993) recently reported a new low-molecular-weight MAP-2 isoform containing four repeats. Whether this repeat promotes MT polymerization remains unresolved. Likewise, MAP-4, originally purified from adrenal cortex, is an ubiquitously expressed protein which has recently been associated with spindle pole bodies and is a member of this MAP superfamily (Olmsted, 1986). It has an acidic N-terminal projection arm domain and a basic C-terminal binding region, similar to MAP-2 and tau. Its projection arm, however, is sequence specific and appears unrelated to

Fig. 1-3: Sequence alignment and homology for tau, MAP-4 and MAP-2. The assembly promoting (AP) sequence for tau, MAP-4 and MAP-2 are given. The daggers and asterisks indicate two or three identical residues among the three MAPs, respectively. Conserved amino acid residues for these regions are shown and the residue numbers are indicated in parentheses.

```
AP sequnce-1           * + * * + * * * + * + * + * * + * *   + + +
TAU:    (263)          V-K-S-K-I-G-S-T-E-N-L-K-H-Q-P-G-G-G-K-V-Q-I
MAP-4:  (881)          V-R-S-K-V-G-S-T-E-N-I-K-H-Q-P-G-G-G-R-A-K-V
MAP-2:  (1674)         V-K-S-K-I-G-S-T-D-N-I-K-Y-Q-P-K-G-G-Q-V-Q-I


AP sequence-2          +                 +         +   +     + + +
TAU:    (294)          V-Q-S-K-C-G-S-K-D-N-I-K-H-V-P-G-G-G-S-V-Q-I
MAP-4:  (914)          V-T-K-A-A-G-P-I-G-N-A-Q-K-P-P-T-G-X-K-V-Q-I
MAP-2:                 -------------------------------------------


AP sequence -3         * + * * * * * +   * *   * + * * * *   * + +
TAU:    (325)          V-T-S-K-C-G-S-L-G-N-I-H-H-K-P-G-G-G-Q-V-E-V
MAP-4:  (950)          V-S-S-K-C-G-S-K-A-N-I-K-H-K-P-G-G-G-D-V-K-I
MAP-2:  (1705)         V-T-S-K-C-G-S-L-K-N-I-R-H-R-P-G-G-G-R-V-K-I


AP sequence -4         + * + * + * * * * *     * + * + * * + + * +
TAU:    (357)          V-Q-S-K-I-G-S-L-D-N-I-T-H-V-P-G-G-G-N-K-K-I
MAP-4:  (982)          A-Q-A-K-V-G-S-L-D-N-V-G-H-L-P-A-G-G-A-V-K-T
MAP-2:  (1737)         A-Q-A-K-V-G-S-L-D-N-A-H-H-V-P-G-G-G-N-V-K-I


CONSENSUS:             V---S-K---G-S-----N-I-K-H---P-G-G-G---V---I
```

the other MAP proteins (Lee *et al.*, 1988). In terms of binding, Aizawa *et al.* (1989) showed the first repeat displaced native MAP-4 from microtubules. In all of these cases, the synthetic peptides bind to MTs with 100-fold weaker affinity suggesting other sequences in the binding region may play a pivotal role in these interactions. Interestingly, all three proteins are rich in proline which may explain their lack of defined structure and rod-like shapes (Voter and Erickson, 1982; Murofushi *et al.*, 1986; Hirokawa *et al.*, 1988).

Neuronal MAP Phosphorylation

Neuronal MAPs are particularly fascinating because of their developmental regulation in both the axon and dendrite. Matus (1988) diagramed MAP-2b as constitutively expressed throughout maturation while the expression of embryonic MAP-2c (axonal) and adult MAP-2a (dendritic) appear alternately synchronized. Similarly, there is a coincident disappearance and appearance of juvenile and adult tau, respectively, in the axon. The data suggest these proteins are involved in determining neuronal shape and regulating the balance between rigidity and plasticity in neuronal processes (Seeds *et al.*, 1970) and the expression of these isoforms may be directly relevant to these functions. As previously noted, MAP-2 and tau appear to stabilize microtubules in a similar manner, but one must remember they are distinctly different proteins. For example, antisense RNA studies revealed a complete loss of neuritic morphology in the absence of MAP-2 (Dinsmore *et al.*, 1991), whereas axonal structure appeared only slightly altered in the absence of tau (Harada *et al.*, 1993). Correspondingly, Chen *et al.* (1992)

transfected SF-9 cells with each protein as well as with MAP-2c and analyzed cross-sectioned MAP-induced processes. The spacing between microtubules was three-fold greater with MAP-2ab (61.7 nm) *versus* either tau (19.8 nm) or MAP-2c (20.8 nm). These findings were consistent with studies of neurons and emphasize the importance of the projection arm domain. Additionally, Yamauchi and Purich (1987) demonstrated a selective association of phosphatidylinositol for MAP-2 *versus* tau proteins.

Protein Kinases

Highly abundant in brain tissue are protein kinases (*e.g.*, cAMP-dependent protein kinase, calcium dependent protein kinases, proline-directed kinases). Goodman *et al.* (1970) were the first to report a protein kinase activity in microtubule protein preparations from bovine brain. Upon MAP phosphorylation MAP-MT interactions are weakened *in vitro* (Jameson and Caplow, 1981; Theurkauf and Vallee, 1983) and extensively phosphorylated species *in vivo* readily dissociate from the microtubule lattice (Brugg and Matus, 1991). Phosphorylated tau proteins, and to a lesser extent MAP-2, have been implicated, through *in vitro* and *in situ* epitope labeling experiments, in the formation of pair-helical filaments (Iqbal *et al.*, 1986; Kosik *et al.*, 1988; Wille *et al.*, 1992b). The kinases which phosphorylate these proteins *in vivo* and ultimately change their binding affinities for microtubules are as yet unresolved. Although these alterations have been inspected by an array of kinases, the physiological relevance of the kinases used is questionable.

Although several protein kinase activities have been co-purified with MAP-2, only protein kinase A (PKA) has a specific binding site on the extreme N-terminus of MAP-2. Obar *et al.* (1990) reported a high affinity (K_d of 5 nM) for the regulatory subunit of PKA for MAP-2. Protein kinase A modifies serine (threonine) residues specifically and exists as a tetrameric holoenzyme: two regulatory subunits that have an antiparallel orientation and two catalytic (Taylor *et al.*, 1990). In brain tissue, both the heart and brain isoforms are present. Leiser *et al.* (1986) showed 80 percent protein kinase A bound to MAP-2 is brain-specific while 20 percent is the heart isoform. The role of the heart isoform and the control provided by tight association of the regulatory subunit with MAP-2 remain unresolved. Deep-etch electron micrographs indicate MAP-2 crosslinks MTs and MT-NF structures (Hirokawa, 1982). This raises the possibility that MAP-2 crosslinks adjacent microtubules *via* the regulatory subunit of PKA. Considering MT-bound MAP-2 displays a lack of susceptibility to proteases, the heart isoform may play a role in maintaining neuronal spacing during increased metabolic activity. This hypothesis remains to be further investigated. Many calcium-dependent cellular processes have been directly coupled to neuronal function. Thus, protein kinase C (PKC), itself a calcium/phospholipid protein kinase, is present in high concentrations in brain tissue and has been implicated in a variety of neuronal signaling pathways. This cellular cascade is complex. Calcium ions enter the cell following depolarization of the presynaptic membrane by an action potential. The influx of calcium triggers an electrical current down the axon, followed by the exocytotic release of various neurotransmitters such as glutamate and acetylcholine. In parallel, PKC becomes stimulated and subsequently modulates ion channels, desensitizes receptors and regulates neurotransmitter release (Nairn *et al.*, 1985). Additionally, PKC can be activated by members of the G-protein cascade. Phospholipases become autophosphorylated on tyrosine residues *via* an extracellular ligand binding. This leads to conformational changes in membranous G-proteins, stimulation of phospholipase C and an increase of diacylglycerol in membranes. Compartmentalized calcium is released and PKC subsequently phosphorylates various substrates.

There are ten isoforms of protein kinase C, falling into three well defined sub-classes: cPKC, nPKC, aPKC (see Table 1-3). PKC has a regulatory and catalytic domain composed of a total of four conserved regions with binding sites for phorbol esters, calcium, ATP, and the substrate of interest. cPKC isoforms were the first group isolated and they display a classical behavior by retaining all four of these regions. nPKC species lack the second domain and are calcium insensitive, while aPKC isoforms were identified for their phorbol ester insensitivity and fatty acid dependence (Tanaka and Nishizuka, 1994). In brain tissue, the cPKC and nPKC isoforms are predominantly observed. Interestingly, PKC expression and activity is elevated during neuritogenesis (Widmer *et al.*, 1993) where MAP-2 is abundant. Additionally, the sustained activation of PKC is apparently needed for neuronal plasticity and regeneration (Hama *et al.*, 1986). Although several *in vitro* studies reveal PKC phosphorylates MAP-2, further investigations *in vivo* are needed.

Table 1-3

Protein kinase C isoforms in mammalian tissues[a]

Isoform	Activators	Tissue expression	Localization (in neurons)
cPKC			
α	Ca^{2+}, DAG, PS	Universal	golgi complex, dendrites
β I	↓	Some	periphery of perikaya
β II	↓	Many	golgi complex, dendrites
γ	↓	Brain, spinal cord	cytoplasmic, nucleus, PM
nPKC			
δ	DAG, PS	Universal	Golgi, axon, nerve terminals
ε	↓	Brain	↓
η	↓	Lung, skin, heart	↓
θ	↓	Skeletal muscle	↓
aPKC			
ξ	PS, FFA	Universal	Not found in brain
λ	unknown	Ovary, tetes	

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[a] Abbreviations: DAG, diacylglycerol; PS, phosphotidylserine; FFA, cis-unsaturated fatty acid; cPKC, classical behavior; nPKC, newly isolated, aPKC, atypical behavior. Compiled from Tanaka and Nishizuka (1994).

Tau proteins

Tau proteins are translated from a single gene, but reducing gel electrophoretic patterns reveal at least six variants (Cleveland *et al.*, 1977). These proteins contain 341 and 441 residues and their relative abundance changes throughout development. Lindwall and Cole (1984) were among the first to observe a phosphorylation-induced electrophoretic mobility shift in these proteins and postulated a potential conformational change. Following alkaline phosphatase treatment, tau proteins assembled to a greater extent than did their phosphorylated forms. These finding have intrigued cytoskeletal researchers and prompted them to identify the kinase(s) inducing this "hyper-phosphorylation" state. Early discoveries by Ihara *et al.* (1983) and Iqbal *et al.* (1986) revealed a hyper-phosphorylated form of tau as a component of Alzheimer paired helical filaments.

Tau has been treated with a variety of physiologically relevant and other protein kinases as a means to study the formation of these aberrant neuronal structures. Tau phosphorylation occurs predominantly on serine and threonine residues. Correlating the specific sites of phosphorylation with changes in cellular function is of especially great interest. Steiner *et al.* (1990) using bacterially expressed tau, deletion mutants and gel electrophoresis indirectly mapped protein kinase action for PKA, PKC, casein kinase and calcium/ calmodulin kinase (CaM) to the binding domain of tau. Unexpectedly, only phosphorylation by CaM kinase showed a pronounced mobility shift characteristic of tau from Alzheimer plaques. Phosphorylation at Ser-405 outside of the repeat motif induced this shift. Interesting work by Lee *et al.* (1991)

isolated a tau-like protein from pair-helical filaments, A68, whose backbone was the same as tau but contained an extra potential phosphorylation site. The authors suggest this alteration converts normal tau into the diseased state. More recently proline-directed kinases such as cdc-2 kinase (Ledesma *et al.*, 1992), MAP kinase (Drewes *et al.*, 1992; Gustke *et al.*, 1992) and cell division kinase 5 (Paudel *et al.*, 1993) have been used to study tau. Although these kinases efficiently phosphorylate tau, their effects on microtubule binding is moderate (Biernat *et al.*, 1993). By contrast, phosphorylation on a single residue, Ser-262 located in the first repeat sequence in the MT-binding domain dramatically alters tau-MT binding interactions. Additionally, recent studies using PKA phosphorylated tau showed a 92 percent decrease in the rate of MT polymerization (Scott *et al.*, 1993). These data suggest that a conformational change in tau proteins may not be the only factor in the formation of these abnormal brain structures.

MAP-2

MAP-2 has three isoforms: MAP-2a, MAP-2b and MAP-2c. A single copy gene encodes MAP-2, but two mRNA transcripts have been detected which arise from alternate splicing (Garner and Matus, 1988). MAP-2c lacks amino acids 147-1519 of the MAP-2 sequence (Kindler *et al.*, 1990) as shown in Figure 1-2 and the difference between MAP-2a and -2b remains to be determined (Binder *et al.*, 1984). Each species has both an N-terminal binding site (amino acids 83-113) for the regulatory subunit of protein kinase A (Rubino *et al.*, 1989) and the previously described MT-binding region. As in the case of tau proteins, MAP-2 phosphorylation occurs on serine and threonine residues, and these modifications have been probed by many kinases (Ray and Sturgill, 1987; Hoshi *et al.*, 1988; Kyriakis and Avruch, 1990). Considerable cytoskeletal research has focused on identifying the importance of phosphorylation on MAP-2 in terms of regulating MAP-MT interactions.

An early series of studies revealed discrepancies in the number of moles of phosphate incorporated per mole of MAP-2. The observed values included two (Sloboda *et al.*, 1975), four (Coughlin *et al.*, 1980) and seven moles (Islam and Burns, 1981). Such differences were attributed to an array of protein kinase activities in brain tissue, including PKA (Theurkauf and Vallee, 1983), CaM kinase (Goldenring *et al.*, 1983), and PKC (Akiyama *et al.*, 1986). Likewise, hydrolysis-resistant endogenous phosphoryls (8-10 sites) are widely known to be present. Tsuyama *et al.* (1987) has since calculated 46 moles of phosphoryl per mole of MAP-2 from microwave-irradiated rat brain tissue. This method minimizes endogenous phosphatase action. Correspondingly, Therukauf and Vallee (1986) showed that the projection arm domain is extensively phosphorylated relative to the MT-binding region. Such phosphorylation patterns have complicated MT-binding analyses where unphosphorylated and phosphorylated MAP-2 were used. The use of kinases and phosphatases to selectively incorporate phosphoryls onto MAP-2 has emphasized that phosphorylation at certain sites may be more influential than others in disrupting or ensuring MAP-MT interactions (Murthy and Flavin, 1983; Brugg and Matus, 1991).

In vivo studies of MAP-2 phosphorylation have yet to be conducted. Recently, several *in situ* studies have observed changes in the phosphorylation of MAP-2 under depolarizing conditions (Diaz-Nido *et al.*, 1993) as well as during the release of neurotransmitters, specifically glutamate (Halpain and Greengard, 1990). These authors suggest that phosphorylation levels are regulated by a calcium-dependent protein kinase, but more data are needed. Recently, incubation with okadaic acid was shown to increase phosphorylation of MAP-2 in cortical neurons (Arias *et al.*, 1993). Intriguingly, this treatment ultimately led to neuronal cell death which the authors suggest was due to a diminished rate in dephosphorylation. Additionally, Diez-Guerra and Avila (1993) observed a parallel between the extent of dendritic arborization and phosphoryl content of MAP-2. These studies again reemphasize the complexity of MAP-2 phosphorylation.

Proposal

MAP-2 is an essential neuronal cytoskeletal component (Dinsome and Solomon, 1991) which dictates microtubule spacing (Chen *et al.*, 1992) and stabilizes microtubules (Sloboda *et al.*, 1976). Phosphorylation has also been shown to regulate MAP-MT interactions. While regions of MAP-2 have been identified as preferable targets for one kinase *versus* another, cytoskeletal researchers have not yet mapped nor identified specific amino acid residues in the MAP-2 sequence where phosphorylation occurs. Additionally, there has been no detailed analysis correlating such sites of modification to the strength of interaction between microtubules and the binding region of MAP-2. It is also unclear whether or not such a disruption is caused by a single phosphoryl or results from concerted influence of several residues. The experiments in this dissertation address these issues using a systematic *in vitro* approach of recombinant protein expression, site-directed mutagenesis and mass spectrometry designed specifically for these studies. Initially, the action of several kinases was explored in terms of ability to modulate the strength of MAP-MT interactions, and protein kinase C was chosen for further investigations. Subsequently, PKC phosphorylation sites in the MT-binding region were pin-pointed, and three pivotal sites influenced MAP-2 and microtubule interactions in terms of binding and polymerization.

CHAPTER 2
EXPERIMENTAL STRATEGIES FOR DEFINING PHOSPHORYLATION SITES

Introduction

The biochemical procedures described within this first section are routinely used to assess protein-protein interactions. These include microtubule binding assays and tubulin self-assembly measurements. Binding assays strictly differentiate the ability of microtubule-associated proteins (MAPs) to bind to a microtubule (MT) lattice under various conditions (*e.g.*, *via* phosphorylation or increasing salt concentrations). MAPs are defined as any protein that remains persistently associated with the microtubule surface and is concentrated upon repeated cycles of warm-induced assembly and cold-induced disassembly. Taxol, a low-molecular-weight natural product of the Yew tree, is known to bind irreversibly to MTs generating a semi-flexible filament (Dye *et al.*, 1993). This agent eliminates all the dynamic properties normally attributed to microtubules (Wilson *et al.*, 1985). This method in conjunction with gel electrophoresis allows the binding affinity to be measured as well as MT-bound and unbound fractions to be analyzed.

Assembly assays examine two separate processes: tubulin nucleation and microtubule elongation. The Varian 210 spectrophotometer is ideal for making these measurements since the detector is positioned far enough away from the light source to reduce any potentially interfering stray light effects. In the presence of fibrous MAPs, nucleation becomes readily stabilized since MAPs bind the MT lattice and hinder the loss of the nucleotide cap at the microtubule ends. Additionally, MAPs propagate the formation of many short microtubule polymers, and they minimize microtubule dynamic instability (Yamauchi *et al.*, 1993). In the absence of the fibrous MAPs, microtubules are not readily formed *in vitro*, and the tubulin must exceed the higher critical concentration (10 µM *versus* 2 µM) in order to self-assemble (Sloboda, *et al.*, 1976; Olmsted, 1986). Electron micrograms of microtubule pools formed in the presence of MAPs reveal that 90-95 percent of the total population are 13 protofilament MTs, and approximately 5 percent consist of 14 protofilament structures (Pierson *et al.*, 1978; McEwen *et al.*, 1980).

The second portion of this chapter outlines methods specifically designed for localization and analysis of phosphoryl acceptor sites in cytoskeletal proteins. MAP-2 is highly phosphorylated, and although so-called recycled MAPs are greatly reduced in their phosphoryl content, they retain substantial levels of endogenous phosphate bound as monoesters. These endogenous phosphoryls can obscure the analysis of phosphoryl-acceptor sites probed with exogenous protein kinases and radiolabeled ATP. Therefore, the use of bacterially expressed recombinant MAP-2 MT-binding region (MTBR) offers an advantage for these studies since the protein is completely unphosphorylated; moreover, the MTBR behaves like native MAP-2 in terms of microtubule interactions. Proteolytic peptide mapping and amino acid sequencing methods are routinely used to identify phosphoryl-acceptor sites. Upon incubation of MAP-2 (both native and recombinant forms) with several proteases (*i.e*, chymotrypsin, endoproteinase lys C, endoproteinase glu C) an overlapping and confusing fragmentation pattern is generated. Accordingly, I engineered specific methionyl residues into the MTBR to produce a defined set of overlapping CNBr fragments of distinct molecular weight. In combination with mass spectrometry and comparison of unphosphorylated and phosphorylated samples, the location of phosphoryl-acceptor sites were assigned. These assignments were later confirmed by site-directed mutagenesis in which I replaced serine residues with alanines and observed a corresponding reduction in radiolabel incorporation.

These techniques provide a general methodology for identifying specific amino acid residues in the MT-binding region that upon phosphorylation modify MAP-MT interactions. Conceivably, this strategy may be applied towards addressing phosphoryl-acceptor sites in the projection arm of MAP-2. Similarly, other cytoskeletal phosphoproteins, other protein kinases as well as other protein modifications can be approached in this manner.

General Methods

Materials

The catalytic subunit of cAMP-dependent protein kinase from bovine heart (P-2645), phosphatidylserine and trifluroacetic acid were purchased from Sigma Chemical Co. (St. Louis). The rat brain isoform of protein kinase C (539494) and its activator (-)-Indolactam V (405272) were obtained from Calbiochem, Inc. (San Diego). Radiolabeled [γ-^{32}P]ATP (Specific Activity $\geq$ 7,000 Ci/mmol) was obtained from ICN

Biomedicals, Inc. (Costa Mesa, CA) along with acrylamide, SDS, ammonium persulfate, bis-acrylamide and Aquacide I. DNAse was purchased from Boehringer-Mannheim (Germany) as a liquid stock as well as fatty-acid free BSA. Phosphocellulose cation exchange resin was obtained from Whatman. Cyanogen bromide as a 5 M liquid stock solution and 3, 5-dimethoxy-4-hydroxy-cinnamic acid (sinapinic acid) were obtained from Aldrich (Milwaukee, WI). All bacterial media components were purchased from Difco. IPTG was obtained from Fisher Biotech (Fair Lawn, NJ). AmpliTaq sequencing kit was obtained from Perkin Elmer Cetus and all restriction enzymes and respective buffers from New England Biolabs. Sequencing grade apomyoglobin was purchased from Beckman Instruments (Irvine, CA). Taxol was received as a gift from the National Cancer Institute (Bethesda, MD). Expression vector pETh3b, a derivative of pET3b described by Studier *et al.* (1990), was generously provided by Dr. Donald R. McCarty of the Department of Vegetable Crops, University of Florida. All chemicals used were of reagent grade.

Purification of Cytoskeletal Proteins

Tubulin. Bovine brain microtubule proteins were prepared by hypotonic extraction as described by Shelanski *et al.* (1973) and modified by Karr *et al.* (1980) through repeated cycles of warm-induced assembly and cold-induced disassembly. Tubulin was isolated by phosphocellulose chromatography in assembly buffer (100 mM Mes, 1 mM EGTA and 1 mM $MgSO_4$ pH 6.8), using 1 ml of resin for every milligram of microtubule protein. Tubulin was then concentrated using an Amicon concentrator, cycled again, and finally frozen in liquid N_2 and stored in small aliquots (30 µl) at -80°C until used. This method routinely yielded MAP-free tubulin with a critical concentration of self-assembly of 8 µM. Tubulin concentrations were determined by Bradford (1976) using BSA as a standard. Calculated tubulin values were multiplied by a correction factor of 0.8.

Native microtubule-associated protein-2. MAP proteins were prepared as described by Herzog and Weber (1978) from microtubule-proteins cycled three times. The homogenized protein solution was boiled for 10 minutes, cooled for 20 minutes and centrifuged at 100,000 x g for 20 minutes at 4°C. MAP-2 was then separated from tau proteins on a BioGel A 1.5-m column in assembly buffer as described by Kim *et al.* (1979). The column (1.5 x 55 cm) was previously packed in assembly buffer at 10 ml/h using a peristaltic pump. The fractions containing MAP-2 were detected using both OD_{280} readings and gel electrophoresis. The 5 ml samples were pooled and concentrated by reverse dialysis against Aquacide I (*i.e.*, carboxymethyl sephadex) at 4°C. Once the desired volume was obtained the protein was transferred to a fresh dialysis bag and dialyzed against assembly buffer overnight at 4°C. The protein was then clarified at 100,000 x g in a Beckman tabletop ultracentrifuge, aliquoted and stored at -80°C until needed. MAP-2 concentrations were determined by Bradford (1976) using BSA as a standard and a correction factor of 3.14.

Recombinant MAPs. The bovine brain cDNA of MAP-2 microtubule binding region (amino acids 1629-1828) was cloned into a pETh3b expression vector under the T_7 polymerase promoter. Upon induction this plasmid, as well as the mutants described, generated either the full-length MAP-2 MTBR or a truncated version (amino acids 1671-1770) designated as N123C and 123 respectively. Such proteins were purified from BL21(DE3) *E. coli* cells as reported, with the minor changes described as follows (R. L. Coffey *et al.*, submitted). Bacterial cultures (typically 1L) were routinely grown in standard LB media (10 mg/ml tryptone, 5 mg/ml yeast extract and 10 mg/ml NaCl pH 7.0) containing 50μg/ml of both ampicillin and chloramphenicol to an OD_{600} of 0.35 at 37°C, induced with IPTG (0.5 mM) and incubated for an additional 2 hours. The cells were then pelleted at 3,500 x g for 5 minutes and washed once with assembly buffer. Following the addition of 10 units/ml of DNAse, the cells were lysed by two passes in the French Press and the resulting slurry was centrifuged at 14,000 x g for 20 minutes at 4°C. The pellet was homogenized in assembly buffer containing 0.75 M NaCl and 1 mM DTT, incubated at 80°C for 10 minutes and centrifuged at 100,000 x g for 20 minutes. This supernatant was diluted 5-fold with cold distilled water, loaded onto a 0.5 ml phosphocellulose column and eluted at room temperature with assembly buffer containing 1 M NaCl. Finally, the MAP protein was concentrated to one-half its original volume using a Savant speed-vac concentrator, dialyzed against assembly buffer and stored at -80°C. All MAP protein concentrations were determined as described by Lowry *et al.* (1951) using BSA as a standard with a correction factor of 0.73 (based on amino acid analysis of MAP-2 wild-type recombinant protein).

Analysis of MAP-MT Interactions

Binding assay to polymerized protein. Unphosphorylated or phosphorylated recombinant MAP protein was prepared as described in the next section. The MAP protein was added at 1.0 µM to varying concentrations of KCl (0-250 mM) in BSA coated airfuge tubes (P30721) prewarmed at 37°C. Taxol-stabilized microtubules were prepared from a 100 µM tubulin stock in the presence of 1 mM GTP and 100 µM taxol in assembly buffer. Subsequently, microtubules were diluted (10 µM) into the MAP mixture (1 µM) and incubated for an additional 20 minutes at 37°C. Samples were centrifuged at 160,000 x g for 20 minutes at 37°C in a Beckman Airfuge to separate MT-bound and unbound fractions. Supernatants and pellets were electrophoresed on a 12% SDS-polyacrylamide gel (Laemmli, 1970) and visualized by Coomassie blue staining and autoradiography. Fractions were quantitated using a BioImage densitometer (model: Visage 60) and the loading was standardized using the tubulin in each lane as an internal control. A 10:1 molar excess of tubulin to MAP was used.

Assembly assay. Various MAP protein mutants (1.0 µM), in their unphosphorylated and phosphorylated form, were mixed with 1 mM GTP, and assembly buffer on ice. Tubulin was fast thawed and added to 10 µM final concentration. The reaction was transferred to a thermostatted cuvette chamber in a Varian 210 spectrophotometer and light scattering, a direct measurement of polymer mass and thus microtubule polymerization, was measured at 350 nm at 37°C.

Methods for Phosphopeptide Localization

Phosphorylation of MAP Proteins

Protein kinase C. Recombinant MAP-2 proteins (20 µM) were incubated with 80 µg/ml phosphatidyl serine (previously sonicated for 10 minutes), 1.5 mM ATP, 1 mM DTT, 0.18 units protein kinase C, 1 µM (-)-indolactam V, in 0.1 ml final volume in PKC buffer (100 mM Mes, 5.5 mM $MgCl_2$ and 1 mM $CaCl_2$ pH 6.2) for specified times (0-16 hours) at room temperature. In stoichiometry determination and sedimentation experiments, radiolabeled [γ-^{32}P]ATP was added at 100,000 cpm total. In binding studies the proteins were not further treated; however, for assembly assays, the phosphorylated material was precipitated with nine volumes of cold acetone overnight at -20°C. This removes residual calcium which is known to inhibit microtubule polymerization. Each sample was then centrifuged at 14,000 RPM for 30 minutes using a Beckman microfuge, dried in a speed-vac, and then resuspended in assembly buffer for analysis. Comparison of acetone treated and untreated unphosphorylated proteins revealed this apparently harsh method did not affect assembly properties.

Protein kinase A. Recombinant MAP proteins (20 µM) were incubated with 1.5 mM ATP, 250 units of protein kinase A, 1 mM DTT and assembly buffer at 37°C for 60 minutes. The kinase was reconstituted in 25 µl DTT (50 mg/ml), incubated at room temperature for 10 minutes and used immediately since the enzyme is active for only 48 hours once resuspended. The phosphorylated protein was directly used in both binding studies and assembly assays. Radiolabeled ATP was added for stoichiometry measurements and binding assays.

Proline-directed kinase. All recombinant MAP proteins (20 μM) were incubated at 30°C with 1.5 mM ATP, 1 mM DTT, 0.2 units of proline-directed kinase, and PDK buffer (50 μM Mops, 0.1 mM EDTA and 10 mM MgAc pH 7.4). The kinase was generously provided by Dr. Jerry Wang from the Department of Medical Biochemistry at the University of Calgary, Alberta, Canada and was isolated as described by Lew *et al.* (1992). Phosphorylated material was used directly in binding assays and the stoichiometry was measured as described below.

In priming experiments (*i.e.*, two sequential phosphorylation reactions) where additive phosphorylation effects were analyzed, proteins were phosphorylated first *via* proline-directed kinase in the presence of non-radiolabeled ATP. The reaction was quenched with 1 mM glucose and 1 unit hexokinase and incubated at 100°C for 2 minutes. The mixture was subsequently phosphorylated *via* either protein kinase C or A in the presence of radiolabeled ATP and the stoichiometry was measured.

Stoichiometry Measurements Using Radioactivity

Whatman 3MM filter paper discs (2.3 cm in diameter) were labeled with a pencil, soaked in 10% (w/v) trichloroacetic acid and air dried. Filters were spotted with 10 μl of phosphorylated reaction mixture and placed into an iced beaker containing cold 10% trichloroacetic acid. After the last time point (4 hours) was taken, the filters were further incubated for 20 minutes and then washed by continuous agitation at room temperature with several changes of the washing solution as described by

Deans *et al.* (1992) and modified as follows. Every 10 minutes the solution was changed into 10% (v/v) trichloroacetic acid (total of five changes), followed by 5% (v/v) trichloroacetic acid (total of three changes), then rinsed with two changes of diethyl ether. The filters were then completely dried using a hair dryer and counted in a Beckman (LS 3801) scintillation counter using the Cherenkov method where $H^{\#} = 0$. Controls included reaction mixtures without enzyme (1) and without MAP protein (2). The total counts per mixture were calculated by spotting and counting 10 µl of complete reaction mixtures without washing the filters.

Site-Directed Mutagenesis

Unique restriction sites (Afl II at position 5106 and Stu I at position 5160) were engineered around the second non-identical repeat in the MT-binding domain of MAP-2, designated m_2, using the Amersham *in vitro* mutagenesis kit. This created a convenient way to rapidly mutate several sites in this region through cassette mutagenesis, a well known technique. The singly and doubly phosphorylation insensitive mutants generated are shown in Table 2-1. Degenerate synthetic oligonucleotides (8 µg, each 55 base pairs) were denatured in 100 mM NaCl at 100°C for 2 minutes and allowed to re-anneal by slowing cooling the sample to room temperature. In conjunction pEth3b vector (800 ng) isolated from DM-1 cells was cut with Afl II and Stu I enzymes and the enzymes were heat inactivated at 65°C for 20 minutes. DM-1 cells were used since they cannot methylate cytosine residues which would normally interfere with Stu I action. The linearized vector was precipitated with 2.5 mM

Table 2-1

Phosphorylation insensitive mutants generated in recombinant MAP-2 binding region

Plasmid [a,b]	Location	Comments [c]
pAA 3	S1711A	protein is N123C-like full-length
pAA 6	T1706A	protein is 123-like
pAA 7	S1707A	123-like
pAA 8	S1711A	123-like
pAA 9	S1706A, S1707A, S1711A	123-like
pAA 14	S1728A	123-like
pAA 15	S1760A	N123C-like
pAA 17	L1713M, L1744M	N123C-like
pAA 18	L1692M, L1702M	N123C-like
pAA 19	S1760A	123-like
pAA 21	L1692M, L1702M	123-like
pAA 22	S2728A	N123C-like
pAA 24	S1703A	N123C-like
pAA 25	S1703A, S1711A	N123C-like
pAA 28	S1703A	123-like

[a] Only plasmids used to localize phosporyl-acceptor sites are listed. Several multiple mutants were made in the second repeat sequence pAA10 - pAA12, not given.

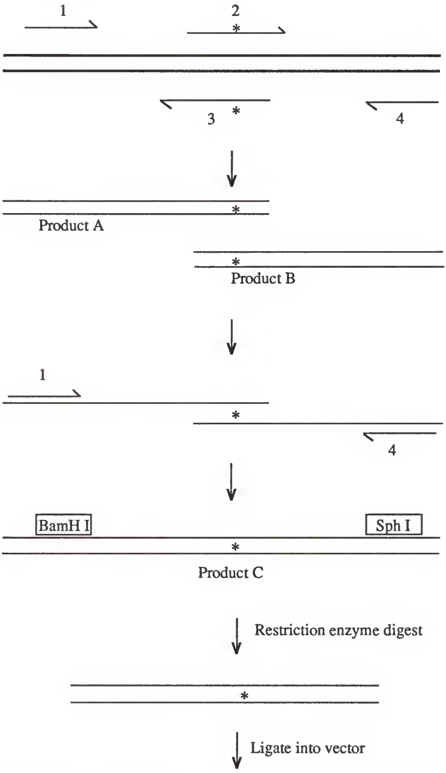
[b] All plasmids contain previously engineered AflII and StuI sites within the pRC8 and pAA4 wild-type constructs containing clones 1629-1828 and 1671-1770, respectively (see Fig. 4-1).

[c] In assembly assays, N123C-like proteins were strictly used. For mass spectral analyses both sized proteins were used, but the truncated version, 123-like proteins, gave consistantly sharper peaks.

ammonium acetate and two volumes of ethanol. Following centrifugation the pellet was dried and resuspended in TE buffer (10 mM Tris-HCl and 1 mM EDTA pH 8.0) at 100 ng/ml. Prepared oligos (2 µg) and vector (800ng) were ligated at 16°C for 4 hours under standard conditions (Sambrook *et al.*, 1989). Following ligation, samples were incubated with Stu I for 1 hour at 37°C, heat inactivated and transformed into competent DM-1 cells. The synthetic oligonucleotides used remove the Stu I recognition sequence, so colonies were screened for the loss of the Stu I site. Each mutant was dideoxy-sequenced according to the AmpliTaq sequencing kit protocol.

Mutants outside this immediate cassette region were produced using PCR mutagenesis. Figure 2-1 shows the overall design of this strategy (Ho *et al.*, 1989). Synthetic DNA primers 2 and 3 were approximately 30 base pairs in length with mismatches positioned near the middle of the primers. These primers were chosen to generate fragments that overlap by at least 20 base pairs which permitted them to be readily annealed. In the first PCR reaction 1-2 ng of plasmid in 74 µl of TE were incubated with the following solution. A master mixture contained: 10x Taq buffer, 0.77 mM ultrapure dNTPs, 5.8 mM $MgCl_2$, 300 pmol of each primer, and 1.5 µl Taq enzyme in 78 µl total volume. One third of this reaction mixture was added to the plasmid to 0.1 ml final volume. Samples were routinely cycled 30 times at 95°C, 42°C and 72°C for 1 minute intervals followed by a final extension period at 72°C for 10 minutes. The initial products were run on a 1.5% low melt agarose gel, stained with ethidium, and gel slices (containing the correct fragment) were further incubated and cycled in a similar manner using primers 1

Fig. 2-1: Four primer PCR strategy for site-directed mutagenesis. Arrows represent the 5′ to 3′ direction. The mutagenesis sites are denoted by asterisks and are placed towards the center for the PCR primer. Products A and B were gel purified from low melt agarose and used to produce product C. The primers are represented as numbers (1-4) and the BamH I and Sph I sites used subsequently for cloning are shown.


1
2
*
*
3
4
*
Product A
*
Product B
1
*
4
BamH I
Sph I
*
Product C
Restriction enzyme digest
*
Ligate into vector

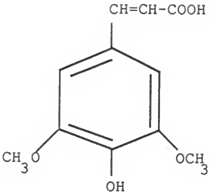
and 4 (see Fig. 2-1) to extend and amplify a 270 base pair region containing convenient restriction sites subsequently used for cloning. The full length product was cut at the internal Bam HI and Sph I restriction sites and ligated into the pEth3b vector cut in a similar fashion. The entire 270 base pair stretch was sequenced using the AmpliTaq sequencing kit to confirm that only the desired mutation(s) were produced.

As a means of specifically localizing phosphoryl acceptor sites in the MT-binding region, PCR techniques were employed to produce convenient sites for chemical cleavage. Specifically, leucine residues were converted to methionines in two combinations, at amino acid positions 1692 and 1702 and at positions 1731 and 1744. These substitutions, in and of themselves, had no effect with respect to interactions with microtubules.

Peptide Fragmentation

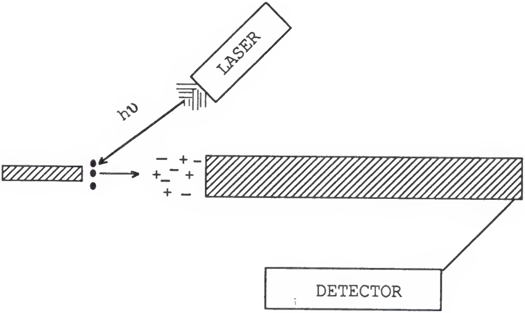
Both unphosphorylated and phosphorylated samples (500 pmol) were cleaved in 70% trifluoroacetic acid using 1-2 mg cyanogen bromide, and incubated in the dark at room temperature for 16 hours. The reaction was then mixed 1:1 with distilled water and the cyanide was removed in a speed-vac and the polypeptide fragments were concentrated to 0.1 ml. The fragments were then acetone precipitated as previously described and resuspended in distilled water. The sample was diluted 1:1 with sinapinic acid matrix to give 5-10 pmol of material for analysis on a time-of-flight mass spectrometer (Vestec, Laser Tec Benchtop II). The structure of sinapinic acid is given in Fig. 2-2 along with a schematic representation of the mass spectrometer. Peaks corresponding to both unphosphorylated and phosphorylated cleavage fragments were compared for shifts of 80 mass units which represent a single phosphoryl group. Doubly and singly charged Apomyoglobin (M_r 17,199) was used to generate a standard curve from which the mass for each MAP fragment was assigned (Chait *et al.*, 1993).

Fig. 2-2: Schematic overview of time-of-flight mass spectrometry. In time-of-flight mass spectrometry the mass resolution ranges from 500 to 66,000 daltons with a 0.01 to 0.2% variability. Once the sample is co-dried with a ionizable matrix component such as sinapinic acid (structure given), the sample is bombarded with a wavelength of 350 nm and becomes ionized. The particles then pass down an electrode tube towards a detector as shown. The detector assigns a mass value to the polypeptide according to the time of travel.



Sinapinic Acid

Time-of-flight Mass Spectrometer



CHAPTER 3
DIFFERENTIAL PHOSPHORYLATION OF MAP-2 BY SEVERAL PROTEIN KINASES

Introduction

The cytoskeleton directly provides an underlying fibrous network for the shape, motility, and structural reorganization of all eukaryotic cells. This is accomplished through the interaction of microtubules, actin filaments, intermediate filaments, and other cytoskeletal proteins. In neurons, maintenance of cellular integrity as well as flexibility is essential for continuous and efficient processing of stimuli traversing from the cell body to terminal synaptic junctions. Discovery of cAMP-stimulated protein kinase in microtubule protein preparations led to initial speculations that second messenger effects may alter microtubule protein function *via* phosphorylation of tubulin or other cytoskeletal proteins (Goodman *et al.*, 1970; Sloboda *et al.*, 1975; Rappaport *et al.*, 1972; Soifer *et al.*, 1972; Eipper, 1972). Nerve growth factor interactions at neurotrophin receptors result in the dramatic reorganization of the neuronal cytoskeleton, and action of this agonist is accomplished, at least in part, by activating protein kinase C (Widmer *et al.*, 1993). Other protein kinase activities are also known to co-purify with microtubule proteins (Vallee, 1980; White *et al.*, 1980; Goldenring *et al.*, 1985).

Beyond the involvement of tubulin, specification of microtubule cytoskeletal structure and function arises from the involvement of microtubule-associated proteins (MAPs). Among these are MAP-2 and tau proteins which both use highly related non-identical repeat motifs to bind to assembled microtubules. Theurkauf and Vallee (1983) demonstrated that MAP-2, a high-molecular-weight, dendritic protein, becomes phosphorylated at numerous sites which weakens its interaction with microtubules. In fact, the deduced amino acid sequence of MAP-2 (Kosik *et al.*, 1988; Dammerman *et al.*, 1989; Lewis *et al.*, 1988) suggests many loci fit the criteria of putative protein kinase A and C phosphoryl-acceptor sites as well as for several proline-directed kinases. Isolation of MAP-2 from rat brain, subjected to focused microwave-irradiation to examine *in vivo* phosphorylation prior to endogenous phosphatase action, revealed 46 covalently bound phosphoryl groups per mole of MAP-2 molecule (Tsuyama *et al.*, 1987). In related studies, Jameson and Caplow (1981) demonstrated that MAP-2 phosphorylation affects both the rate and extent of microtubule polymerization *in vitro*, and Raffaelli *et al.* (1992) found that MT dynamic instability is altered under similar conditions. Nonetheless, surprisingly little is known about the location of actual phosphorylated sites on the MAP-2 molecule, and even less is known about those sites that influence the strength of MAP-2 binding to microtubules directly.

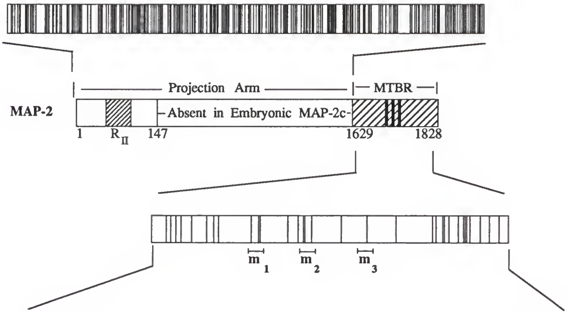
It is noteworthy that MAP-2 consists of at least two different functional domains: the MT-binding region (MTBR) which interacts directly with microtubules; and the projection-arm which extends away from microtubules and may serve a cross-linking role (Vallee and Borisy, 1979; Flynn *et al.* 1987). The greatest extent of MAP-2 phosphorylation was evident in the projection-arm region as opposed to the smaller 22-35 kD microtubule-binding region (Theurakauf and Vallee, 1983). Thus, efforts to analyze changes in MAP-2 affinity for microtubules on the basis of the stoichiometry of phosphorylation in the entire MAP have proven ambiguous. Since the MT-binding region, obtained by thrombin cleavage of MAP-2, retained virtually all the binding strength of the intact protein (Flynn *et al.*, 1987; Joly *et al.*, 1989), I accordingly have focused my efforts on understanding the regulatory role of MAP-2 MTBR phosphorylation on MT binding. I have utilized the bacterially expressed MT-binding region and several kinases as biochemical tools.

Results

MAP-2 is especially rich in serine and threonine residues that represent potential sites for phosphorylation (indicated as vertical lines in the diagram shown in Fig. 3-1). Phosphorylation on this molecule has been probed with various kinases both *in vitro* and *in vivo*, some of which have no psychological relevance (Theurkauf and Vallee, 1983; Akiyama *et al.*, 1986; Hoshi *et al.*, 1992). I experimented initially with protein kinase A (PKA) which Sloboda *et al.* (1975) found to bind directly to the projection arm of MAP-2 and has a K_d of 5 nM (Leiser *et al.*, 1990). Additionally, protein kinase A has been shown to influence MAP-MT interactions in experiments using recycled MAP-2 protein (Jameson and Caplow, 1981; Brugg and Matus, 1991; Raffaelli *et al.*, 1992). Interestingly, it is the only kinase to date shown to directly associate with MAP-2.

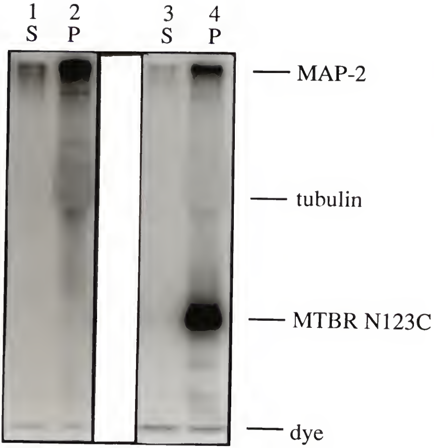
Initial binding studies however using protein kinase A and recycled bovine-brain MAP-2 proved ambiguous. Figure 3-2 exhibits both

Fig. 3-1: Schematic representation of potential phosphorylation sites. The expanded sections display the positions of serine and threonine residues (vertical lines), which represent 17% of the total amino acid composition of MAP-2 and emphasize the abundance of potential sites for modification. The projection arm and MT-binding region contain 288 and 31 potential phosphoryl-acceptor sites respectively. The amino acid sequence for the previously characterized bovine-brain MAP-2 MTBR is given with the three non-identical repeat sequences highlighted (Coffey *et al.*, submitted). The human MAP-2 cDNA sequence (Kosik *et al.*, 1988; Dammerman *et al.*, 1989) was used for the placement of serine and threonine sites in the projection arm, and the amino acid alignment was correlated with the mouse cDNA (Lewis *et al.*, 1988).



```
    HTP
1629-T PGTPKSAILV PSEKKVAIIR TPPKSPATPK QLRLINQPLP DLKNVKSKIG
  1680-STDNIKYQPK GGQVQIVTKK IDLSHVTSKC GSLKNIRHRP GGGRVKIESV
  1730-KLDFKEKAQA KVGSLDNAHH VPGGGNVKID SQKLNFREHA KARVDHGAEI
  1780-ITQSPGRSSV ASPRRLSNVS SSGSINLLES PQLATLAEDV TAALAKQGL
```

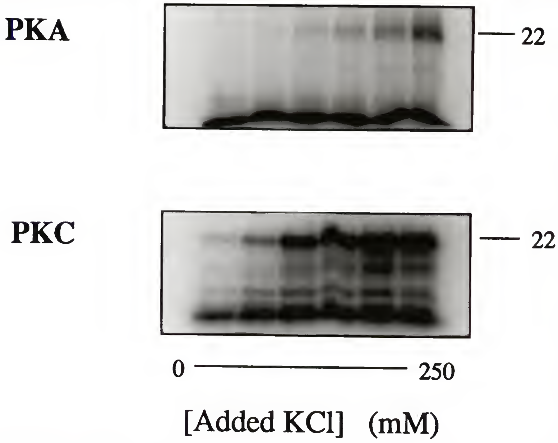
Fig. 3-2: Differential effects of phosphorylation on MT binding with native and recombinant MAP-2. Autoradiogram of supernatant and pellet fractions after native MAP-2 or MTBR-N123C treatment with protein kinase A for 1 hour at 30°C. MT-unbound (S) and bound (P) fractions were separated in the presence of brain MAP-2 alone (lanes 1 and 2, respectively) and in the presence of brain MAP-2 and recombinant protein (lanes 3 and 4). Both types of MAP proteins were phosphorylated separately prior to incubation with taxol-stabilized MTs. M_r values (kD): MAP-2, 200; tubulin, 55; MTBR, 22.


1
S
2
P
3
S
4
P
MAP-2
tubulin
MTBR N123C
dye

the supernatant and pellet fractions following ultracentrifugation to separate MT-unbound (S) and bound (P) fractions respectively, using either native MAP-2 alone (lanes 1 and 2) or in the presence of recombinant MAP-2 MTBR (lanes 3 and 4). While phosphorylation by protein kinase A of intact MAP-2 (200 kD) weakened MAP interactions with the microtubule lattice, the recombinant protein (22 kD) remained associated. A 10 molar excess of taxol-stabilized microtubules was used to ensure binding sites for both MAP species; however as seen in lane 1 (Fig. 3-2), MAP-2 is dissociated upon phosphorylation in the absence of MTBR. The native protein was selected for its ability to bind MTs and this criteria thereby rejected any MAP-2 molecules which were extensively phosphorylated in the MTBR, while the recombinant MAP-2 was not phosphorylated by bacterial protein kinases. This suggested that there may be an additive effect among endogenously and exogenously added phosphoryl groups. Brugg and Matus (1991) demonstrated that a sub-population of endogenous phosphoryls are insensitive to calf alkaline phosphatase and indeed the binding capacity of native MAP-2 treated in this manner followed by phosphorylation was unchanged (data not shown).

Earlier work demonstrated that 0.35 M KCl is necessary to dissociate MAP-2 MTBR from microtubules (Joly *et al.*, 1989). I surmised that phosphorylation occurring at or near a critical binding site would increase this sensitivity to salt-induced desorption. In terms of binding (Fig. 3-3, top panel), incubation with protein kinase A resulted in no apparent change in MAP-MT binding and the binding region was dissociated from the microtubule lattice only upon relatively high levels of KCl (200-250 mM). Phosphorylation *via* protein kinase A

Fig. 3-3: Comparison of MAP-MT binding as regulated by protein kinases A and C action. Autoradiogram of MT-unbound fractions after MTBR-N123C treatment with either PKA or PKC for 1 hour as indicated. KCl was added in increments of 50 mM, ranging from 0 to 250 mM final. A 10:1 molar excess of tubulin to MAP was used. Coomassie stained 12% gels reveal a similar pattern with equal protein loading per lane. The lower bands in the gel correspond to incomplete translation products rather than random proteolysis or *E. coli* protein contaminants. They share a common N-terminal sequence as demonstrated through western blot analysis (Y. Zhang, personal communication). These polypeptides appear not to influence the apparent binding properties of full-length MTBR to microtubules when compared to high-pressure-liquid chromatography purified recombinant protein, which eliminates these smaller sized products.

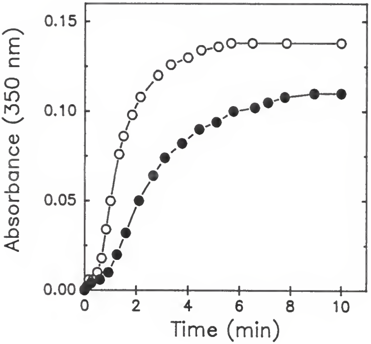

PKA
22
PKC
22
0
250
[Added KCl] (mM)

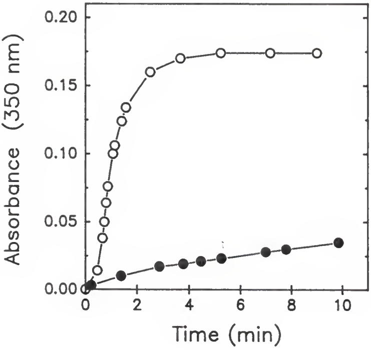
however may affect microtubule dynamics in another manner. Indeed in assembly assays, protein kinase A action altered the ability of MAP-2 MTBR to stabilize microtubule nucleation and lowered the overall extent of assembly (Fig. 3-4A). Although an *in vitro* effect was seen, these changes were modest. The rate of polymerization was retained to 80 percent as compared to the unphosphorylated protein and likewise, 45 percent of the overall extent was preserved. Therefore, I decided to probe these interactions further utilizing several other kinases.

Based on the differential effects of unphosphorylated and phosphorylated second-repeat peptides to displace tau proteins from microtubules, Correas *et al.* (1992) reported that phosphorylation of this peptide *via* protein kinase C (PKC) could reduce the affinity of tau for microtubules. Accordingly, I was interested to see if protein kinase C, isolated from brain tissue, phosphorylated MAP-2 MTBR at a similar site and how this might alter MAP-MT interactions. As shown in Figure 3-3 (bottom panel) and Figure 3-4B, protein kinase C induced a significant change in both the binding and assembly properties of the MAP-2 MT-binding region to microtubules. Upon phosphorylation by this kinase there was an immediate release of MTBR from the microtubule lattice, with optimal dissociation occurring under physiological ionic conditions (*i.e.*, 0.12-0.15 M KCl). The assembly properties were also altered dramatically, with only 4 percent of the rate of polymerization being retained and 24 percent of the overall extent of assembly relative to the unphosphorylated protein (Fig. 3-4B).

In related studies two proline-directed kinases were utilized to explore this region further. These kinases target serine-proline and

Fig. 3-4: Comparison of differential assembly properties with MAP-2 MTBR-N123C *via* phosphorylation by protein kinases A and C. The change of absorbance *versus* time was measured as a direct measurement of microtubule polymerization. MAP-2 MTBR-N123C protein (1.5 μM) was phosphorylated for 4 hours with either PKA or PKC as indicated and incubated with 10 μM phosphocellulose purified tubulin. Unphosphorylated (open symbols) and phosphorylated (closed symbols) samples were compared.


Protein kinase A


Protein kinase C

threonine-proline amino acid motifs. MAP-2 contains many of these amino acid combinations and *in vitro* assays demonstrate that cell cycle and cell division kinases phosphorylate MAP-2 (Hoshi *et al.*, 1992). Although this is an intriguing finding, neurons do not undergo cell division and MAP-2 therefore would not be affected by such kinases. I chose to experiment with brain-specific, non-mitotic kinases from this family which may mimic MAP-2 phosphorylation *in vivo*. Lew *et al.* (1992) characterized proline-directed kinase (PDK) from brain homogenates and showed that it is distinct from the cell cycle kinases and indirect immunofluorescence data revealed its localization in the cytoplasm (J. H. Wang, personal communication). Recently PDK was cloned, bacterially expressed and renamed cyclin division kinase-5 (cdk-5). I measured radiolabel incorporation by PDK and calculated 9 mol of phosphoryl per mol of MAP molecule. Although PDK extensively phosphorylates the MAP-2 MT-binding region relative to protein kinase A and C (Fig. 3-5) with 4 and 7 mol of phosphoryl per mol of MAP molecule respectively, there was no apparent influence on MAP-MT binding (see 30 minute lane, Fig. 3-6). Upon further incubation (60 and 120 minutes) and more phosphoryl incorporation (12 mol phosphoryl per mol MTBR), the MAP is not released from the microtubule lattice. Similarly, phosphorylation *via* extracellular regulated kinase I or II, which co-purify with native MAP-2, did not influence the interaction of MAP-2 MTBR with microtubules (J. M. Angelastro, personal communication).

Sequence analysis of the MT-binding region revealed several consensus sequence motifs (Table 3-1) for PKA, PKC and PDK. Interestingly, all the proline-directed kinase sites are outside of the

Fig. 3-5: Phosphoryl incorporation on MAP-2 MTBR using radiolabeled ATP and three distinct kinases. MTBR-N123C wild-type protein was phosphorylated with either protein kinase A or C or proline-directed kinase in the presence of radiolabeled ATP as described [*see chapter 2*]. The radiolabel incorporation over time was measured by spotting presoaked TCA filters followed by various washings to minimize unincorporated radiolabel contamination. The range of values is shown with n = 3.

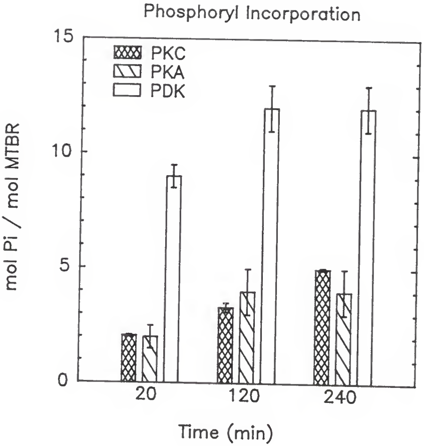

Phosphoryl Incorporation
15
10
5
0
PKC
PKA
PDK
mol Pi / mol MTBR
20
120
240
Time (min)

Fig. 3-6: Displacement of MAP-2 MTBR *via* proline-directed kinase phosphorylation. MAP-2 MTBR-N123C was phosphorylated with PDK as described [*see chapter 2*] for 0, 30, 60 and 120 minutes. Following treatment the MAP sample was incubated with assembled tubulin (10 µM) in the absence of added salt and centrifuged at 30°C. Shown are the supernatant (top panel) and pellet (bottom panel) fractions representing MT-unbound and bound treated MAP protein respectively.

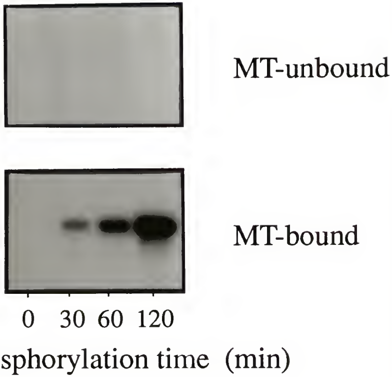

MT-unbound
MT-bound
0
30
60
120
Phosphorylation time (min)

Table 3-1

Consensus phosphorylation sites: Specificity motifs

Protein kinase	Motif[a]	Frequncy[b]
cAMP-dependent	**R**-X-S	21/46
	R-**R**-X-S	12/46
	R-X-X-S	11/46
	K-**R**-X-X-S	2/46
calcium / phospholipid dependent (PKC)	S/T-X-**K/R**	20/37
	K/R-X-X-X-S/T	13/37
	K/R-X-X-S/T-X-**K/R**	7/37
	K/R-X-S/T	10/37
	K/R-X-S/T-X-**K/R**	6/37
proline-directed[c]	P-X-S/T-X-**R/K**	undefined

[a] Specificity determinants are shown in bold type (Pearson and Kemp, 1991).

[b] These values were generated from a random sampling of phosphoproteins and does not necessarily hold true for MAP-2. Structural and biochemical properties may alter these values.

[c] Brain-specific proline-directed kinase, recently renamed cdk-5, is a an apparently promiscuous enzyme since it phosphorylates many more sites in MAP-2 MTBR than predicted.

non-identical repeat region while both protein kinase A and C share many potential phosphoryl-acceptor sites in this region. In light of my initial findings with native MAP-2, I was motivated to explore potential secondary effects of proline-directed kinase on protein kinase A and C action. These experiments however were fruitless, revealing that neither PKA/PKC phosphoryl incorporation nor MAP-MT binding was altered (data not shown).

Discussion

Phosphorylation acts as a molecular switch for the regulation of various cellular processes ranging from glycolysis to the cell cycle. In a similar manner, phosphorylation of structural proteins may lead to changes in cellular morphology and function. Microtubule-associated protein-2 (MAP-2) in its underphosphorylated form, is known to be involved in mediating vesicular transport, regulating microtubule dynamics, and maintaining dendritic structures (Kowalski and Williams, 1993; Dinsmore and Solomon, 1991). Accordingly phosphorylation of MAP-2 affects these processes (Jameson and Caplow, 1981; Brugg and Matus, 1991). Interestingly, amino acid sequence analysis reveals 17 percent of the molecule consists of serine and threonine residues which are potential sites of phosphorylation. In fact, investigations on kinases often use MAP-2 as a substrate to measure enzymatic activity because MAP-2 contains various specific consensus sequence motifs.

Tsuyama *et al.* (1987) isolated MAP-2 by microwave irradiation and estimated 46 phosphoryl groups on this molecule. This method minimizes endogenous phosphatase action and increases the likelihood of analyzing phosphorylation under *in vivo* conditions. Conceivably, potentially more phosphoryls are on the MAP-2 molecule which were not detected due to the irreversibility of such modifications. Until recently cytoskeletal researchers have focused their attention on the cell biological phenomena triggered by MAP-2 phosphorylation rather than on the identification of specific sites of phosphorylation (Jameson and Caplow, 1981; Drewes *et al.*, 1992). Consequently, sites of phosphorylation have not been correlated to particular changes in cellular function. Such biochemical analyses have been complicated by the overwhelming extent of modification on this protein. With recent advances in protein chemistry and molecular biology, biochemical analyses are more feasible and may prove fruitful.

The experiments presented in this chapter were designed to identify a brain-isolated protein kinase which would diffuse MAP-MT interactions in an *in vitro* system. Although several *in vitro* studies using recycled native MAP-2 identify specific kinases whose action weaken MAP-MT associations, conclusions from such data are tenuous in light of potential interference by endogenous phosphoryls (Brugg and Matus, 1991). Following phosphatase treatment, a sub-population of these phosphoryls remain covalently bound to the protein at unidentified loci (Brugg and Matus, 1991). Therefore, bacterially expressed recombinant protein methods are optimal for addressing protein modifications, such as phosphorylation, because the bacterium does not usually post-translationally alter the over-expressed protein of interest.

From the kinases studied in this section, protein kinase C phosphorylation of MAP-2 led to the most significant changes in terms of MAP binding and assembly with microtubules (see Fig. 3-3 and Fig. 3-4). Interestingly, protein kinase C is activated during neuritogenesis (Widmer *et al.*, 1993), a process during which MAP-2 is highly abundant (Matus, 1988). Whether protein kinase C affects MT-dynamics *in vivo* remains to be determined, but this preliminary set of experiments suggests that this kinase can be used as a probe to identify specific sites of modification in the MT-binding region. I hypothesize that protein kinase C phosphorylates at or near sites that are critical points of interaction between MAP-2 and microtubules. Although the overall random coil structure of MAP-2 is predicted from electron microscopy work (Voter and Erickson, 1982), nothing is known about the detailed molecular structure of this molecule. Therefore, the investigations described in chapter 4 concerning protein kinase C address consensus sequence sites. Serine and threonine residues in the second non-identical repeated sequence are of particular interest (see Fig. 3-1) since Joly *et al.* (1990) demonstrated that the peptide competed with MAP-2 in binding assays and promoted assembly. However, this peptide displayed a 100-fold weaker binding affinity for MTs as compared to native MAP-2. This indicates that other areas of the MAP-2 MT-binding region may play a significant role in MAP-MT interactions and seemingly so may phosphorylation in these regions.

I investigated MAP-2 phosphorylation in terms of alterations in MT-dynamics but phosphorylation is known to regulate other cellular processes. Conceivably, non-protein kinase C modified sites in MTBR may influence numerous other mechanisms and it is difficult to imagine them not participating in some type of control process. Flynn *et al.* (1987) found that MAP-2 interacts with neurofilament L (NF-L) through this common C-terminal region. The amino acid residues that interact with NF-L are not known and accordingly the sites of phosphorylation which disrupt MAP-(NF-L) interactions have not been defined. Sedimentation assays in the presence of phosphorylated MTBR may elucidate the role of modification at these other sites. Additionally, MAP-2 phosphorylation may influence organellar transport down the microtubule lattice and some of these PKA and PDK sites may regulate these interactions.

Interestingly, protein kinase A has a more promiscuous consensus sequence motif when compared to the action of protein kinase C (Table 3-1). Sequence analysis suggests that protein kinase A should modify all the serine and threonine residues which protein kinase C phosphorylates, however my data indicate this is not the case (Fig. 3-5). It is noteworthy that in these studies the heart isoform of PKA was used to phosphorylate MTBR. Although the heart isoform comprises only 20 percent of the PKA found in brain tissue, Leiser *et al.* (1986) showed it preferentially binds to MAP-2 *versus* the brain isoform. Additionally, the heart isoform of PKA has a longer half-life in brain tissue relative to the brain isoform (Stein *et al.*, 1987). Perhaps exploring this region with the brain isoform, would phosphorylate the MT-binding region adequately and thus evoke a measurable effect. Interestingly, preliminary data suggests that cleavage by thrombin of a longer recombinant MAP-2 protein (amino acid residues 1509-1828), which includes the so-called hinge region (see Fig. 1-1), is diminished upon protein kinase A phosphorylation.

With regards to proline-directed kinases, initial interest stemmed from studies using phosphorylated tau proteins which revealed the accumulation of aberrant antiparallel structures and a complete loss of neuronal morphology (Iqbal *et al.*, 1986; Lang *et al.*, 1992; Wille *et al.*, 1992a). Interestingly, Biernat *et al.* (1993) demonstrated that a single phosphoryl within the microtubule-binding region of tau led to paired helical filament formation and Alzheimer-like plaques. Since MAP-2 is extremely homologous to tau proteins in this region and also has been identified in these plaques and can form antiparallel structures *in vitro* (Wille *et al.*, 1992b), I was motivated to determine any effects of these kinases on MAP-MT interactions. As the data indicate, no *in vitro* control is apparent. This does not preclude the possibility that this enzyme regulates MAP-2 *in vivo*; however, in terms of PDK directly controlling MAP-MT interactions, this seems unlikely.

CHAPTER 4
CONTROL OF MAP-MT INTERACTIONS VIA PROTEIN KINASE C

Introduction

Several investigations have described rapid phosphorylation of MAP-2 at multiple sites, as characterized by both physiologically relevant and other unrelated protein kinases (Franklin *et al.*, 1993; Drewes *et al.*, 1992; Hoshi *et al.*, 1992). Despite numerous reports, nothing is known about the specific sites and nature of these modifications towards MAP-MT interactions. Interestingly, Tsuyama *et al.* (1986) uncovered differential behavior on MAP-2 by various protein kinases (*i.e.*, PKC, PKA, and calcium/calmodulin protein kinase). MAP-2, radiolabeled *in vivo* and subsequently treated with alkaline phosphatase *in vitro*, was preferentially re-phosphorylated only by protein kinase C. They concluded that this finding indicates a likely *in vivo* role for PKC on the high-molecular-weight protein. Additionally, Akiyama *et al.* (1986) used two-dimensional gel electrophoresis to show that protein kinase C recognizes additional and unique sites on MAP-2 as compared to protein kinase A. Protein kinase C action, in these experiments, weakened the interaction of MAP-2 with actin microfilaments. Interestingly, protein kinase C is highly active during neurite outgrowth, particularly throughout the formation of dendritic processes where the production of MAP-2 is seemingly most active (Matus, 1988). Hoshi *et al.* (1988) also showed that protein kinase C phosphorylation of the native MAP-2 MT-binding region altered both the rate and extent of microtubule assembly. These analyses are consistent with my studies using the recombinant MAP-2 MT-binding region (see chapter 3).

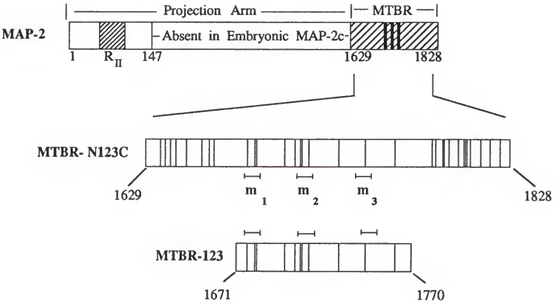
I have concentrated my efforts on identifying those protein kinase C phosphorylation sites located in the microtubule-binding region of MAP-2. This proteolytic fragment retains virtually all the binding energy for microtubules as that observed with the intact molecule. Although I wanted to identify all PKC phosphoryl-acceptor sites in this region, those amino acid residues that were critically affecting MAP-MT interactions were of great interest. Specific attention was given to any modifications occurring within the second non-identical repeat that is known to compete with MAP-2 and promote microtubule assembly (Joly *et al.*, 1989; Joly and Purich, 1990). I used bacterially expressed MAP-2 MT-binding region, containing newly introduced methionyl residues, to readily localize phosphorylation sites. Overlapping partial chemically cleaved fragments were generated. Furthermore, I confirmed that these sites directly influence MAP binding to microtubules in subsequent site-directed mutagenesis experiments converting phosphoryl-acceptor sites to alanyl residues thus blocking subsequent phosphorylation.

I report here the localization and the role of two specific serine residues (Ser-1703 and Ser-1711), situated between the first and second repeat and within the second non-identical sequence repeat, in terms of protein kinase C-mediated phosphorylation control of MAP-microtubule interactions. This investigation also uncovered evidence for the involvement of a third phosphorylated serine residue (Ser-1728) whose action is conditionally dependent on the phosphorylation state of one or both of the above mentioned sites. My data imply that the *in vivo* binding of MAP-2 to microtubules may be regulated by kinase action at these residues.

Results

MAP-2 is known to be highly phosphorylated (Theurkauf and Vallee, 1983; Tsuyama *et al.*, 1987), a finding in agreement with sequence analysis suggesting numerous potential phosphorylation sites by protein kinase C. Nonetheless, extensive phosphorylation known to occur in the projection arm have thwarted identification of specific phosphoryl acceptor sites associated with alterations in MAP-MT interactions. Upon incubation with thrombin, native MAP-2 can be separated into two principal domains: the projection arm (190 kD) and the MT-binding region (22 kD). Flynn *et al.* (1987) and Joly *et al.* (1989) demonstrated that MAP-2 microtubule-binding region (MTBR) retains all the binding affinity for microtubules of the intact protein. For these reasons and to eliminate spurious results arising from endogenous phosphoryls in MAP-2 isolated from brain tissue, I chose to continue to investigate those phosphorylation sites occurring within the microtubule-binding domain using recombinant cDNA expression. As shown in Figure 4-1, I chose to work with two MTBR plasmids, designated here as N123C and 123, with the N representing the sequence from the thrombin cleavage site (position 1629) to the three non-identical peptide repeats (numbered 123) and C representing the sequence following those repeats to the carboxyl terminus (position 1828). Although MTBR-123 has a 4-fold weaker binding affinity for microtubules as compared to the full length

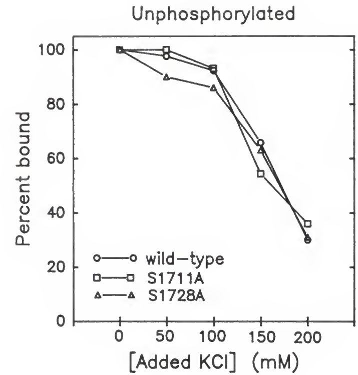
Fig. 4-1: Illustration of different MAP-2 MTBR constructs. Two constructs of the MT-binding region (MTBR), designated N123C and 123 were used in subsequent studies. They share the non-identical repeated motif (m_1, m_2 and m_3) and contain 31 and 11 potential phosphoryl-acceptor sites respectively. Serine and threonine residues are shown as vertical lines. MTBR-123 has a 4-fold weaker binding affinity for microtubules relative to the full-length MT-binding region. However, in companion studies this smaller protein facilitates analysis of N-terminal and C-terminal phosphorylation sites by eliminating them.


Projection Arm
MTBR
MAP-2
R_{II}
Absent in Embryonic MAP-2c
1
147
1629
1828
MTBR- N123C
1629
m_1
m_2
m_3
1828
MTBR-123
1671
1770

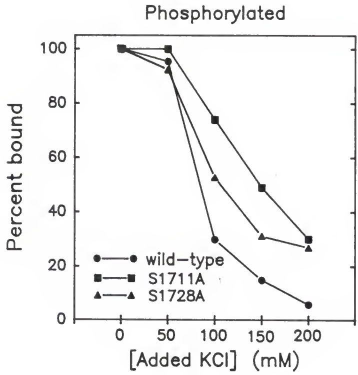
microtubule-binding region, in companion experiments it allows the three non-identical repeat motif to be inspected more carefully. Additionally, eliminating several potential phosphorylation sites at the N- and C-terminal portions of the protein can be valuable, when looked at in conjunction with the full-length MTBR.

In preliminary studies, I mutated the MTBR-123 plasmid to eliminate potential protein kinase C phosphoryl-acceptor sites. Since no structural data are available for either the intact MAP-2 or the MT-binding region, I chose to initially alter amino acids that strictly held the PKC consensus sequences, as inferred from previous studies of other protein substrates (Pearson and Kemp, 1991). Bacterially expressed MAP proteins were incubated with ATP in the absence or presence of protein kinase C, incubated with taxol-stabilized MTs, and following centrifugation, unbound and MT-bound fractions were visualized after electrophoresis. The pelleted fractions were quantitated by densitometry and plotted as the percent of initial bound as illustrated in Figure 4-2. Increasing amounts of salt were added to magnify the sensitivity of binding interactions. Amino acid substitutions at Ser-1711 (in the second repeated sequence) and Ser-1728 (between the second and third repeat) do not alter the apparent binding affinity of MAP-123 to MTs relative to the wild-type protein. However, following phosphorylation with PKC, the amount of MAP protein remaining bound to the MT lattice changes. At 100 mM added KCl, where the differences are most evident, nearly 80 percent of the S1711A and 55 percent of the S1728A mutant proteins pellet with the microtubules, whereas only 25 percent of the wild-type, which is fully phosphorylated, remains

Fig. 4-2: Analysis of MAP-2 binding to microtubules using protein kinase C consensus sequence specific mutants. Protein kinase C consensus sequence sites within the MTBR-123 were mutated to block potential phosphorylation. Purified proteins were incubated in the presence (closed symbols) or absence (open symbols) of PKC [*see chapter 2*]. Following subsequent incubation with taxol-stabilized MTs, the MT-bound fractions were electrophoresed, visualized by Coomassie staining and quantitated using densitometry. All values are calculated relative to 100% initial bound in the presence of zero added salt. Refer to Figure 3-1 for location of each mutant. Wild-type (○, ●); S1711A (□, ■); S1728A (△, ▲).



Unphosphorylated



Phosphorylated

associated. These data suggest that Ser-1711 and Ser-1728 are phosphorylated. Correspondingly, modification at these sites contribute at least in part to dissociating MAP-2 from the microtubule surface, since specifically eliminating phosphorylation enables the MAP to remain bound.

In order to identify phosphoryl-acceptor sites more directly, peptide mapping techniques were utilized. Nonetheless, efforts using standard methods to produce suitable proteolytic fragments proved unsuccessful, a result likely to be related to the highly basic (pI = 10.2-10.5) nature of the MT-binding region (Flynn *et al.*, 1987). This region also lacks methionyl and tryptophanyl residues and contains only a single cysteinyl residue, thereby limiting chemical cleavage alternatives. Therefore, I used site-directed mutagenesis to introduce methionyl residues at four different locations within and flanking the non-identical repeats chosen to produce overlapping cyanogen bromide fragments. I designed plasmids to include pairs of methionines in order to maximize the number of overlapping fragments produced by incomplete CNBr fragmentation, as shown in Figure 4-3. The predicted and observed molecular masses are shown for the unphosphorylated protein, and the values are well within the reproducibility of the Vestec laser-desorption time-of-flight mass spectrometer (Chait and Kent, 1992). Representative mass spectral data are shown in Figure 4-4 for untreated protein as well as that phosphorylated for 4 hours in the presence of protein kinase C. Molecular masses for phosphorylated fragments were found to be increased by multiples of 80, the mass of a single phosphoryl group. Table 4-1 summarizes the time-course of

Fig. 4-3: Calculated and observed mass/charge ratios for MTBR phosphopeptides. Overlapping peptides generated upon CNBr cleavage of methionyl-containing MTBR constructs is shown [*see chapter 2*], with consensus sequence-specific PKC phosphorylation sites indicated with vertical lines. Note that not all of these sites were modified. Each unphosphorylated fragment correlates to a specific molecular weight (predicted) and is comparable to the value obtained from the Vestec mass spectrometer (observed), where m = mass and z = unit charge. The variability in these values was self-consistent for a particular fragment in both the unphosphorylated and phosphorylated form, suggesting that certain peptides potentially contained matrix adducts which shifted the mass. Variability ranged from 0.1-1.5% with n > 10. Note that not all desired peptides were generated or desorbed by the instrument and the data given are for fragments that were reproducibility generated. Unphosphorylated samples were run as a control with each experiment.

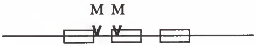
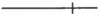
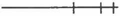
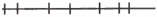
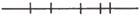
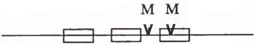
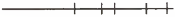
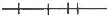
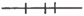
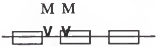
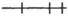
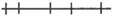
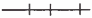
Fragment	**Diagrammatic Localization**	**M/z** **(predicted)**	**M/z** **(observed)**
N123C		21,989	22,012
18-1		8,411	8,518
18-2		9,382	9,400
18-3		13,279	13,320
18-4		12,309	12,280
N123C		21,989	22,012
17-1		12,911	13,020
17-3		10,152	10,215
17-4		8,779	8,769
123		10,924	10,945
21-1		3,595	3,538
21-2		4,565	4,571
21-3		7,368	7,380
21-4		6,398	6,313

Fig. 4-4: Representative mass spectra for unphosphorylated and phosphorylated MTBR 123. Shown are two mass spectral tracings for PKC phosphorylation time points, 0 and 240 minutes. Each peak (marked with a plus) corresponds to a fragment produced from the MTBR-123 construct, as detailed in Fig. 4-3 and *chapter 2* with the molecular weight labeled above (n = 4). Unmarked peaks represent either doubly charged fragments or peptides shifted by matrix adducts.

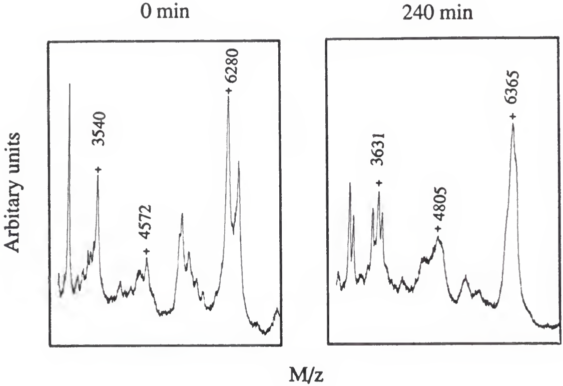

0 min
240 min
Arbitary units
3540
4572
6280
3631
4805
6365
M/z

Table 4-1

Phosphorylation time-course of MAP-2 MT-binding region by protein kinase C

	Phosphoryl Incorporation (mol/mol)			
Fragment	20 min	120 min	240 min	960 min
123	2[a]	3	4	5
21-1	---	0	1	1
21-2	2	2	3	3
21-3	2	3	---	---
21-4	0	1	1	2

[a] Only interger values are reported because laser desorption mass spectrometry does not permit quantitative analysis of the absolute amounts of protein species.

phosphorylation of the so-called MTBR-123 region of MAP-2, and these findings allowed me to localize the phosphoryl groups to serines at positions 1680, 1703, 1711, 1728, and 1760 (Fig. 4-5). Each assignment was confirmed by individual site-directed mutants of serine to alanine which were phosphorylated and evaluated by mass spectroscopy to demonstrate decreased phosphoryl incorporation. In addition to these sites, N123C contained 2 moles P_i in the C-terminal region, but these did not alter MT binding or assembly properties of the N123C plasmid (data not shown).

It is noteworthy to reiterate that the second non-identical repeat promotes tubulin polymerization (Joly *et al.*, 1989) and MAP-2 release from MTs (Joly and Purich, 1990); likewise, the sequence between the first and second repeats in tau proteins also promote tubulin polymerization (Goode and Feinstein, 1994). Accordingly, I focused my attention on positions Ser-1703 and Ser-1711 in terms of phosphorylation effects on tubulin polymerization. The findings shown in Figure 4-6 were obtained with wild-type N123C as well as single and double mutations leading to alanine substitutions at 1703 and 1711. Phosphorylation of the wild-type N123C by PKC reduced the polymerization rate to 22 percent of unphosphorylated protein without altering the extent of assembly. Single mutants behaved similarly with an inability to stimulate tubulin polymerization after phosphorylation. However, the double mutant S1703A/S1711A retained full potency in stimulating MT self-assembly, even when exposed to protein kinase C phosphorylation for 20 minutes. This result indicates that modification at either Ser-1703 or Ser-1711 is apparently sufficient to weaken microtubule MAP-2

Fig. 4-5: Diagrammatic representation of protein kinase C sites in MAP-2 MT-binding region. The amino acid position for each phosphoryl was determined from overlapping partial cleavage products over time (see Fig 4-3 and Table I). 5 mol/mol was calculated for MTBR-123 and 7 mol/mol for MTBR-N123C while locations were confirmed to be identical in this shared region. Radiolabeled stoichiometry measurements were made using phosphorylation insensitive mutants (ser to ala) at each position to confirm values and the reduction of radiolabel was observed (data not shown).

Localization of PKC sites in MTBR-123 Construct

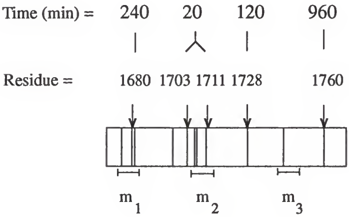
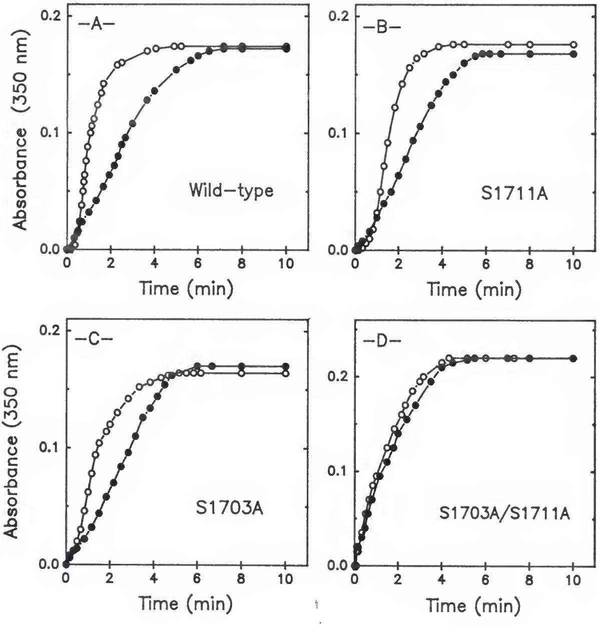


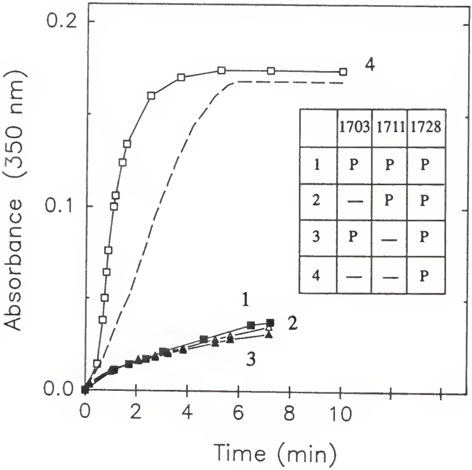
Fig. 4-6: Influence of Ser-1703 and Ser-1711 phosphorylation on MT assembly kinetics. Each panel displays turbidity *versus* time as a measurement of tubulin polymerization (10 µM) in the presence of 1.0 µM MTBR-N123C either (O) unphosphorylated or (●) phosphorylated with PKC for 20 minutes [*see chapter 2*]. *A:* wild-type; *B:* S1711A; *C:* S1703A; *D:* S1703A/S1711A.


-A-
Wild-type
-B-
S1711A
-C-
S1703A
-D-
S1703A/S1711A
Absorbance (350 nm)
Time (min)
0.0
0.1
0.2
0
2
4
6
8
10

interactions. Mass spectrometry confirmed the unit stoichiometry of phosphorylation with the single mutants and the lack of detectable phosphorylation with the double mutant.

Finally, I studied the role of phosphorylation at positions 1703 and 1711 in respect to phosphorylation at other sites. I measured the ability of wild-type and mutant N123C to stimulate MT assembly after 2 hours phosphorylation by PKC (Fig. 4-7) because, earlier experiments (see Fig. 4-2) revealed phosphorylation at Ser-1728 significantly weakened the strength of MAP binding to taxol-stabilized microtubules. The dashed line shows the assembly-stimulating properties of wild-type N123C at 20 minutes phosphorylation (data from Fig. 4-6A), whereas curve 1 indicates that both assembly rate and extent of the same protein are greatly suppressed after 2 hours incubation with protein kinase C. Curves 2 and 3 show that the single mutants S1703A and S1711A are similarly affected by more extensive covalent modification by the kinase. In all three cases, the site of the observed additional phosphorylation was localized to Ser-1728 by mass spectrometry after cyanogen bromide cleavage (see inset to Fig. 4-7). Significantly different behavior was discovered with the double mutant S1703A/S1711A which acts like the unphosphorylated wild-type, while still retaining a phosphoryl at position 1728. These results strongly suggest that while phosphorylation at position-1728 can reduce both MAP's ability to stimulate the rate and extent of MT assembly, such an effect is conditional with respect to modification at 1703 or 1711.

Fig. 4-7: Influence of Ser-1728 phosphorylation on MT assembly kinetics. MTBR-N123C constructs were incubated for 2 hours with PKC [*see chapter 2*] and assayed as described in Fig 6. In the inset (P) denotes the position of phosphoryl incorporation, whereas (-) indicates the absence of a phosphoryl resulting from a serine to alanine mutation. The dashed line represents phosphorylation of wild-type for 20 minutes as a point of reference (data from Fig 6). *Curve 1* (■): wild-type; *curve 2* (▲): S1703A; *curve 3* (▴): S1711A; *curve 4* (□): S1703A/S1711A.


Absorbance (350 nm)
0.0
0.1
0.2
Time (min)
0
2
4
6
8
10
1
2
3
4
1703
1711
1728
1 P P P
2 — P P
3 P — P
4 — — P

Discussion

Fibrous MAPs, such as MAP-2 and tau, contain numerous sites of phosphorylation that are likely to play various regulatory roles in the neuronal cytoskeleton (Hirokawa, 1982; Wiche, 1989; Matus, 1990). MAP-2 is known to interact with microtubules and intermediate filaments *in vitro* (Aamodt and Williams, 1984; Flynn and Purich, 1987), and MAP phosphorylation modifies the strength of MAP-microtubule binding (Jameson and Caplow, 1981; Hoshi *et al.*, 1988; Raffaelli *et al.* 1992). The organization of the MT cytoskeleton is also likely to require facile local release of MAP cross-bridges to allow unimpeded organellar transport *via* dynein and kinesin motors. Likewise, the intracellular location of MAP-2 follows a developmentally specified program involving MAP-2c in embryonic and neonatal axons and the high-molecular-weight forms MAP-2 (MAP-2ab) in the dendrites of central nervous system neurons (Wiche, 1989; Matus, 1990). Whether MAP phosphorylation controls any or all of these functions remains to be elucidated, but one can be confident that strategies for localizing specific sites of phosphorylation will facilitate future studies of kinase-mediated control.

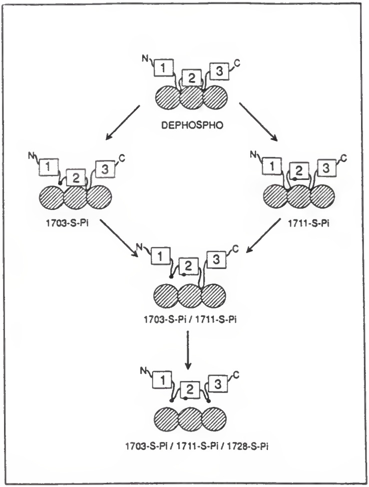
My experimental approach of introducing methionyl residues to generate a useful set of overlapping fragments after incomplete CNBr treatment should be applicable in future investigations of the multi-functional domain structure of MAP-2. Analyzing MAP-2c should be particularly feasible since it contains only 300 additional residues more than the microtubule-binding region itself. The deduced amino acid sequence of rat MAP-2c, for instance, indicates that there is only a single internal methionine in the entire molecule, and cloning of the cDNA corresponding to the N-terminal 160 amino acids of bovine MAP-2c should afford a means for producing recombinant MAP-2c. Eventually, one would wish to investigate those phosphorylation sites located within the much longer projection arm of high-molecular-weight MAP-2ab, because these sites are likely to influence the spacing of microtubules in dendrites (Hirokawa, 1982). Such an endeavor promises to be most challenging, given the earlier findings of Theurkauf and Vallee (1983) and Tsuyama *et al.* (1987) demonstrating extensive phosphorylation in the projection arm.

In principal protein-protein interactions such as those between MAP-2 and one of its macromolecular ligands can be regulated by phosphorylation of MAP-2 or its binding counterpart. My work has concentrated on MAP-2 interactions with microtubules, but interactions with such proteins as neurofilaments could be altered as a result of MAP-2 phosphorylation or the well known phosphorylation of all three neurofilament proteins. Likewise, the N-terminal part of MAP-2 contains a binding site for the regulatory subunit of type II-β cAMP-dependent protein kinase. Keryer *et al.* (1993) recently demonstrated that phosphorylation of the regulatory subunit by cyclin B/p34^{cdc2} decreases the affinity of MAP-2 for the regulatory subunit. While the potential regulatory significance of this phosphorylation reaction remains to be elucidated, site-directed mutagenesis offers a means for analyzing those MAP-2 amino side-chain groups that are involved in this binding interaction.

I chose to explore phosphorylation of the MAP-2 MT-binding region in terms of subsequent effects on MAP-stimulated tubulin polymerization. I found that short-term exposure of the recombinant protein to PKC altered the rate of polymerization but not the final extent of assembly. This result may involve effects on either nucleation or elongation, but factors affecting elongation rates usually have some effect on the protomer-polymer equilibrium and are evident as changes in the overall extent of assembly (Purich and Kristofferson, 1984). Alternatively, I could have chosen to examine MAP phosphorylation in the presence of assembled microtubules. In the latter case, the dynamics of MAP association and dissociation from MTs could influence the susceptibility of serine and threonine sites on the binding region to protein kinase C action. Depending on the nature of conformational changes within the MT-binding region in its bound and free states, sites other than those identified in these experiments may be found to undergo phosphorylation. Likewise, agents that disrupt the interactions of MAP-2 with microtubules, such as phosphatidylinositol (Yamauchi and Purich, 1987), may alter the ability of MAP-2 to become phosphorylated. Such an example was reported very recently by Brandt *et al.* (1994) who studied the phosphorylation of bacterially expressed human tau protein by protein kinase A in the absence or presence of heparin, an acidic antagonist of MT binding to microtubule-associated proteins possessing non-identical sequence repeat motifs. They found that heparin bound to tau in a manner that affected phosphorylation and subsequent tubulin polymerization by modified tau. They concluded that the activities of tau in promoting the growth of assembled tubules as well as nucleation of new MTs are differentially affected by specific phosphoryl sites on tau.

Based on the differential effects of phosphorylated and unphosphorylated second-repeat peptides to displace tau from microtubules, Correas *et al.* (1992) first reported that the phosphorylation in the second peptide repeat of tau could reduce the affinity of this microtubule-associated protein for microtubules. Their assignment of Ser-313 in tau protein as the putative phosphorylation site influencing MAP-MT interactions corresponds to my confirmed assignment of phosphorylation at Ser-1711 in MAP-2. In related studies on tau protein, Goode and Feinstein (1994) recently reported that the so-called inter-repeat sequence between the first and second non-identical repeats in tau is capable of promoting microtubule self-assembly. They also found that deletion of this region from tau proteins greatly reduced the strength of tau binding to microtubules *in vitro*. My finding that phosphorylation at Ser-1703 lowers the efficiency of the MAP-2 MTBR-stimulated tubulin assembly is in basic agreement with their corresponding studies on tau. Based on their schematic representation of the interaction of the MT-binding region with an assembled microtubule, one can consider the diagram shown in Figure 4-8. Here the first inter-repeat and the second repeat make strongest contacts with the microtubule lattice. Since the MAP-2 MT-binding region can bind to assembled microtubules with a stoichiometry of one MTBR-N123C per tubulin dimer (R. L. Coffey and D. L. Purich, submitted), this diagram does not include interactions of the first and third repeats with neighboring tubulin subunits in the microtubule

Fig. 4-8: Diagrammatic representation on interactions of MAP-2 MTBR with microtubules. The shaded circles represent tubulin subunits, and the squares correspond to the three non-identical repeats in the MAP-2 MT-binding domain. Strong interactions are indicated by the contact made between the first inter-repeat, the second non-identical repeat, and the third inter-repeat. Weak interactions for first and third repeats are indicated by the lack of contact by these repeats with the tubulin subunits. This model suggests why phosphorylation at Ser-1728 cannot abolish MAP-MT interactions unless phosphoryls are also present at positions 1703 and 1728.


N
1
2
3
C
DEPHOSPHO
1703-S-Pi
1711-S-Pi
1703-S-Pi / 1711-S-Pi
1703-S-Pi / 1711-S-Pi / 1728-S-Pi

lattice. An interaction of the third inter-repeat with microtubules is also included to account for the fact that phosphorylation at Ser-1728 can greatly weaken MAP-2 binding. To my knowledge, my results are the first to demonstrate contributions of the first and third inter-repeat to the binding energy released upon MAP-2 complexation with microtubules. Since one must still rely upon such diagrams rather than having a detailed three-dimensional structure of MAP-2 only emphasizes the need for more fine structural information on this protein. For example, the structural basis for the observed conditional effects of phosphorylation at Ser-1728, itself located in the second inter-repeat, cannot as yet be accessed in terms of its interactions with either Ser-1703 or Ser-1711. Phosphorylation at Ser-1728 takes place with the same time course, irrespective of phosphorylation at either Ser-1703 or Ser-1711, as indicated by site-directed mutants lacking phosphoryl acceptor sites at 1703 and/or 1711.

I have observed that protein kinase C phosphorylation of the MAP-2 MT-binding region does not result in complete dissociation of the MAP from microtubules, at least not under the salt conditions typically employed in microtubule assembly experiments. Furthermore, distinct differences in the binding behavior of the MTBR have been identified, depending on the extent of phosphorylation. These considerations may help to explain the complexity of binding interactions recently reported by Wallis *et al.* (1993), especially when one considers that their MAP-2 preparations were undefined with respect to the extent of phosphorylation. Moreover, MAP-2 derived from whole brain tissue preparations is apt to display microheterogeneity in terms of phosphorylation, and this may limit quantitative analysis of its interactions with microtubules. Ongoing experiments in the laboratory interestingly indicate that unphosphorylated MAP-2 MTBR shows no evidence of cooperativity in MT binding (R. L. Coffey and D. L. Purich, submitted).

These experiments have focused on *in vitro* interactions of MAPs with microtubules, and one cannot be certain of the relevance of these findings to the cellular behavior of MAP-2 in neurons. While one must be mindful of this caveat, transfection experiments using MAP-2 MTBR plasmids containing methionyl residues could help to assess the sites of intracellular MAP phosphorylation. The high sensitivity of mass spectrometry should also facilitate such studies, especially with the arrival of state-of-the-commercial-art signal intensifiers requiring only picomole amounts of proteins.

CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

Phosphorylation is a regulatory process that nowadays seems to be mentioned in almost every seminar. It acts as a molecular switch in terms of limiting a series of cellular events from replication to glycolysis to the cell cycle. Intriguing reports suggest that transcription is controlled by extensive phosphorylation at the carboxyl terminus of RNA polymerase II which subsequently inhibits enzyme-DNA interactions (Baskaran *et al.*, 1993). Likewise, histone proteins that are transiently modified on serine residues have been found to dissociate from tightly wound DNA throughout replication. While no correlation with chromatin function is known, these findings are intriguing. Additionally, phosphorylation on transmembrane receptors triggers second messenger cascades which lead to subsequent activation of several protein kinases (*e.g.*, protein kinase C and calcium/calmodulin protein kinase) as well as several oncogenes (Mathews and van Holde, 1989). In terms of structural macromolecules, phosphorylation is known (a) to alter protein conformation (Drewes *et al.*, 1992), (b) to disrupt protein-protein interactions (Jameson and Caplow, 1981), and (c) to favor the formation of aberrant cellular structures in Alzheimer's disease (Iqbal *et al.*, 1986).

Microtubule-associated protein-2 (MAP-2) is an essential component of the neuronal cytoskeletal architecture. When MAP-2 biosynthesis is suppressed by the use of antisense oligonucleotides, dendritic processes are lost (Dinsmore and Solomon, 1991). Aside from MAP-2 being developmentally regulated (Matus, 1988), phosphorylation apparently attenuates the strength of MAP-2 interactions with microtubules (MTs) (Jameson and Caplow, 1981; Raffaelli *et al.*, 1992). Although significant changes in stimulated microtubule assembly by MAP-2 have been reported for the phosphorylated protein, no one has determined the sites of specific kinase-mediated modification, much less connected these modifications to altered cellular function. The complexity of MAP-2, a multi-functional domain protein capable of interacting with MTs, NFs and microfilaments, has frustrated such analyses. Until now, no practical nor feasible strategy has been proposed and executed to approach these goals. Although the studies presented in this dissertation use *in vitro* protocols to address cytoskeletal interactions, these techniques conceivably can be readily applied to studies *in vivo*. The objective of this dissertation has been to identify amino acid residues in the microtubule-binding region of MAP-2 that undergo phosphorylation and result in alterations of MAP-MT interactions. The experiments described in this document emphasize how one may approach the systematic biochemical identification and functional analysis of these sites of covalent modification.

Native MAP-2 is routinely purified by repeated cycles of warm-induced assembly and cold-induced disassembly along with other microtubule-associated components and tubulin (Weingarten *et al.*, 1975). In this manner only those phosphorylated forms of MAP-2 that retain their MT binding properties are isolated. More extensively modified forms are probably lost from these preparations during centrifugation of assembled tubules. Investigators have assumed that endogenous phosphoryls on MAP-2 selected in this manner do not interfere with further *in vitro* analyses. However, as shown in Figure 3-2, there is an apparent difference in the binding capacities between recycled MAP-2 and bacterially expressed MAP-2 subsequently phosphorylated by protein kinase A. The use of recombinant protein expression therefore represents an ideal substrate for addressing these or other types of protein modifications. Moreover the bacterium does not post-translationally modify MAP-2 as demonstrated by my extensive database of mass spectral determinations. Thus, modifications by a particular kinase can be assayed with respect to microtubule binding, self-assembly or vesicular transport.

In addition to using recombinant expression methods, specific sites for chemical cleavage were incorporated into MAP-2 to facilitate localizing phosphoryl-acceptor sites (Fig. 4-3). An advantage of placing a total of four methionines, two at a time, into two separate plasmids was to provide the opportunity to analyze small regions of the MTBR sequence. Additionally, this minimized an undefined mass spectral phenomenon known as peak suppression. Under some unknown conditions, certain peaks will be omitted from the signal; whereas, if these fragments are analyzed individually corresponding peaks can be obtained. Furthermore, because cyanogen bromide (CNBr) rarely cleaves proteins completely, the fragments were reproducibly generated. With two mutants (leucine converted to methionine), I increased the likelihood that all portions of the molecule were analyzed. I designed this method to simplify peptide mapping techniques which commonly rely on amino acid sequence-specific proteases. A common problem with using proteolytic enzymes is that they frequently recognize additional secondary sites for cleavage. Often, ambiguous polypeptide maps can be difficult to evaluate, particularly when analyzing a protein with repetitive sequences such as MAP-2. Cyanogen bromide cuts directly at known sites producing fragments of predictable molecular weight (see Fig. 4-3).

Peptide mapping and amino acid sequencing are standard methods for localizing phosphoryl-acceptor sites. Although widely used and accepted, a problem with this latter technique is that each PTH-derivative has an UV spectrum used in detection of products of Edman degradation which diminishes with each residue cycle, and phosphorylated residues generate no signal whatsoever. A weak signal representing a unphosphorylated amino acid can therefore be misinterpreted as a phosphorylated residue. To avoid these potential discrepancies, I used mass spectrometry to evaluate chemically generated fragments. A laser-desorption time-of-flight mass spectrometer shows two peaks per fragment, appearing as mass/charge on the horizontal axis at positions corresponding to a singly and doubly charged species. The first represents the true mass value, and the second corresponds to one-half the mass value respectively. Comparison of unphosphorylated and phosphorylated protein fragments, in conjunction with site-directed mutants, allows the precise localization of phosphoryl-acceptor sites without inferences.

Using these methods, I identified seven serine residues in the MT-binding region (MTBR) of MAP-2 which are phosphorylated by PKC. Three of these sites were characterized in terms of their ability to alter MAP-MT interactions as assessed by MT binding and MT assembly assays. Protein kinase C proved useful in my *in vitro* biochemical experiments, but whether PKC has any actual physiological action on MAP-2 remains to be determined. Additionally, whether these are the sites of modification *in vivo* and if indeed PKC (or another kinase) leads to phosphorylation-regulated changes in MT dynamics is yet another unchartered research area.

I believe the next logical step is to study MAP-2 phosphorylation *in vivo* using cultured neurons such as chicken-derived dorsal root ganglion cells or rat primary culture cerebellar neurons. If carried out with MAP-2c in an appropriate vector, the protocols developed in my work would facilitate differentiation of endogenous MAP-2c (lacking methionine sites) from plasmid-derived MAP-2c. Ideally, cultured cells could be transfected with the methionine-containing MT-binding region cDNA. Following protein expression and post-translational modification, MAP-2c will be isolated and treated with thrombin to generated the MTBR for further analysis. In order to ensure that MAP-2 in its various phosphorylated states is isolated, direct heat treatment of lysed cells may be used. At this step, two proteins would be obtained: tau proteins (63 kD) and the MAP-2 MTBR protein (22 kD). As observed with brain homogenates, no other heat-stable proteins should be present. These proteins could then be separated using a series of gel filtration chromatography columns. Once the full-length MTBR is isolated, phosphoryl-acceptor sites can be determined using mass spectrometry techniques. If the same sites are modified as previously determined *in* *vitro* then gross morphological, cellular changes should be analyzed. In the lab, another bacterial plasmid contains the MT-binding region with an additional 21 base pairs on the 5′ end of the clone. The corresponding amino acid sequence is antigenic for the monoclonal T7 polymerase antibody. Stably transfected neuronal cells or fibroblast cells that are microinjected with the MTBR protein (processed in neurons and re-isolated) can be methanol fixed and immunoflorescently stained to localize the epitope tagged protein.

Correspondingly, *in situ* phosphorylation studies may reveal that different amino acid residues are being modified other than those found *in vitro* throughout this dissertation. Presuming this is the case, subsequent *in vitro* binding and assembly experiments should be done to determine how microtubule dynamics differ with MAP-2 modifications at these other sites *versus* those described in this document. Differential interference contrast (DIC) microscopy should be used in the *in vitro* studies to provide better visualization. DIC also allows dynamic properties of a single microtubule to be measured directly in terms of nucleation and elongation rates (Kowalski and Williams, 1993). Additionally, this technique can monitor sudden changes in polymer length and stability fluctuations in the presence of unphosphorylated and phosphorylated MAP-2 MTBR.

Kinase(s) that modify MAP-2 are also of great interest in the cytoskeletal research community. There are reasonable arguments why different kinases may play a role in regulating MAP-MT interactions in regards to the localization of the kinase (Sloboda *et al.*, 1975; Taylor *et al.*, 1990) as well as evidence of their action from *in vitro* analyses

(White *et al.*, 1980; Jameson and Caplow, 1981; Hoshi *et al.*, 1988). Cytoskeletal investigators suggest that the cell cycle-like kinases are the most intriguing in light of current research on tau proteins and pair helical filament formation. However, it is difficult to imagine that only one kinase modifies MAP-2 *in vivo*, especially in light of 46 moles of esterified phosphate in isolated MAP-2 samples (Tsuyama *et al.*, 1987). Interestingly, protein kinase C is present at high concentrations in neurons and has been implicated in numerous neuronal functions including neurite growth, regeneration and plasticity (Tanaka and Nishizuka, 1994). Since the recognition motifs of protein kinases differ significantly, the action of several groups of kinases can be preferentially blocked in cultured cells distinguishing protein kinases A *versus* C modified sites. After treating the cells in this manner, MTBR can be subsequently isolated, cyanogen bromide cleaved and subjected to mass spectrometry. Additionally, analysis of normal *versus* metabolically stressed cells may provide clues to why MAP-2 phosphorylation occurs to different extents thus creating a heterogenesis population. The potential of protein kinase up-regulation over time as an effect on MAP-2 phosphorylation is an intriguing possibility.

Conceivably, such regions of MAP-2 as the binding domain, the projection arm and the hinge region may be targeted by different kinases. My experience indicates that while protein kinase C affects interactions between the MT-binding region of MAP-2 and microtubules, preliminary studies with protein kinase A reveal protease inaccessibility upon phosphorylation in the hinge region. Whether protein kinase A induces conformational changes in MAP-2 has not been explored. By utilizing the CNBr-cleavable MTBR proteins and perhaps introducing one other methionine N-terminus of the thrombin cleavage site, one could begin to inspect phosphoryl-acceptor sites in this region after *in vitro* or *in situ* phosphorylation. If future experiments reaffirm this observed protease inaccessibility, sedimentation velocity ultracentrifugation studies could be used to obtain evidence for conformational changes in the MAP molecule. Either the longer MT-binding region polypeptide (1509-1828, including the hinge region) or recombinant bovine/human MAP-2c (600 total amino acids) in the unphosphorylated and PKA phosphorylated forms should be used. Likewise, these experiments could be employed to investigate other phosphorylated states of MAP-2, such as during antiparallel dimer formation.

With advances in molecular biology technique, antisense RNA experiments and knockout experiments are potentially interesting avenues to pursue. Dinsmore and Solomon (1991) used antisense MAP-2 RNA and demonstrated the complete loss of dendritic processes. Correspondingly, Caceres *et al*. (1992) used SF-9 cultured cells to introduce the formation of neurites. Because their work showed that MAP-2c was sufficient to induce neurite outgrowth, I propose that the MAP-2 MT-binding region will act in a similar manner. If processes form, then one can address the structural changes, if any, in the presence of phosphorylated MAP-2. Protein kinase C is known to be abundant during neuritogenesis, and one must learn whether this contributes to flexible neuronal structures such that the number or strength of MAP-2 cross-bridges are diminished.

The methionine-containing MTBR protein additionally can be used to find other MAP-2 protein interactions. Heimann *et al.* (1985) first demonstrated that MAP-2 binds to neurofilaments, particularly NF-L. Flynn *et al.* (1987) localized the binding affinity to the MTBR from the thrombin cleavage site. While the second non-identical repeat in MAP-2 is known to interact with microtubules, nothing is known about the amino residues in this 22 kD region which associate with the NF-L. In combination with preliminary competition binding assays using synthetic peptides for m_1, m_2 and m_3, CNBr-generated peptides may be useful to analyze interactions outside the three repeat region. Polypeptide fragments can be collected by HPLC or capillary electrophoresis. Capillary electrophoresis can be applied to isolate these peptides followed by analysis on the mass spectrometer to identify each fragment. A potential obstacle in this procedure is ultimately recovering enough material for subsequent studies, but since MAP-2 MTBR is bacterially expressed and relatively easy to isolate, this can be overcome. Additionally the phosphoryl-acceptor sites in the binding region of MAP-2 affecting MAP-NF interactions can be identified using the protocols described in this dissertation as a guideline.

Paschal *et al.* (1989) showed that dynein movement can be stopped upon the addition of MAP-2. The authors suggest that MAP-2 directly modulates microtubule-based motility *in vivo*. Proteolytic treatment and site-directed mutagenesis on α and β tubulin implicated a four amino acid stretch (E-G-E-E) where both MAP-2 and dynein bind on the microtubule lattice. Recently, Hagiwara *et al.* (1994) confirmed these findings using a separate set of techniques. The role of phosphorylation in regards to MAP-2 regulation of motor activity down microtubules is of interest. Using the techniques described in this dissertation, the sites of phosphorylation can be localized.

In closing, it is noteworthy that the exact role of MAP-2 phosphorylation in neurons remains to be elucidated. Nevertheless, the protocols and findings in this dissertation should facilitate future investigations towards understanding cytoskeletal interactions *in vivo*.

APPENDIX
CHARACTERIZATION OF HIV PROTEINASE ACTION ON MAP-2

Introduction

The neuronal cellular cytoskeleton contains numerous microtubules (MTs) running along axons and dendrites. These supramolecular structures are cross-linked to each other and to other structural components (Hirokawa *et al.*, 1988). Several biochemical and cell biological investigations provide evidence suggesting microtubule-associated proteins (MAPs) may modulate these interactions (Purich and Kristofferson, 1984; Olmsted, 1986). The fibrous MAPs, are known to copolymerize with tubulin through cycles of warm-induced assembly and cold-induced disassembly, and they are of particular relevance to MT stabilization. The MAP-2 family of proteins is especially abundant in adult dendritic processes (Binder *et al.*, 1985; Garner *et al.*, 1988) and anti-sense RNA studies reveal a direct dependence of dendritic integrity on MAP-2 (Dinsmore and Solomon, 1991). Alternate splicing of the MAP-2 mRNA leads to the formation of MAP-2ab (a 280 kD dendritic doublet adult form) and MAP-2c (a 70 kD axonal embryonic form). Each of these isoforms shares an identical C-terminal MT-binding region as well as a 150 amino acid stretch at the N-terminus (Papandrikopoulou *et al.*, 1989). This high degree of conservation suggests a potential cross-linking role for MAP-2c in the axon as well.

MAP-2 readily undergoes proteolysis *in vitro* in the presence of trypsin and chymotrypsin to transiently form a 240 kD projection arm domain and a 35 kD microtubule-binding region (Vallee, 1980). Joly *et al.* (1989) also showed that thrombin action on MAP-2 resulted in the stable accumulation of these two components. All these cleavages occur within the so-called hinge region of MAP-2. Tau proteins, which are themselves highly homologous to MAP-2 (Lewis *et al.*, 1988), have a similar cleavage site. Indeed, Aizawa *et al.* (1988) first reported this for tau and carried out amino acid sequencing of chymotryptic fragments. Preliminary findings on MAP-2 in our laboratory also suggest that phosphorylation may regulate proteolysis in this region (R. S. Parekh, A. M. Ainsztein and D. L. Purich, unpublished findings).

The proteolytic susceptibility of MAP-2 *in vitro* has suggested that microtubule interactions *in vivo* could be affected by such cleavage in ways that could alter cross-linking, the spacing of filaments, and even interactions with other cellular organelles. Interestingly, several recent investigations have shown an abundance of thrombin and chymotrypsin protease inhibitors in brain tissue (Festoff *et al.*, 1992; Hook *et al.*, 1993). Additionally, Matus and Green (1987) revealed an age-related increase in levels of a cathepsin D-like protease in rat brain that degrades MAP-2 at near-neutral pH when cells are broken. Several other reports reveal calpain-mediated proteolysis of MAP-2 (Fischer *et al.*, 1991; Johnson *et al.*, 1991).

Microtubules from Alzheimer's patient brain are defective in assembly (Iqbal *et al.*, 1986), and such tissue showed evidence of accumulated MAP-2 antigens in the cell body and proximal dendrites

(Kosik *et al*., 1984). This suggests a potential degradation of the MT-binding region perhaps *via* proteolysis. I also was motivated to study the action of the HIV proteinase, after autopsies of brain tissue from AIDS patients revealed the accumulation of Alzheimer-like plaques. Using purified bovine-brain MAP-2, I focused on characterizing proteolytic polypeptide fragments in terms of microtubule binding and assembly. While these studies were reaching completion, Wallin *et al*. (1990) observed that retroviral proteinases such human immunodeficiency virus (HIV) type-1 cleaved unfractionated microtubule proteins. Subsequently, my studies were published in the Journal of Neurochemistry (Ainsztein and Purich, 1991).

Methods

Cytoskeletal proteins. Bovine brain microtubule proteins (*i.e.*, tubulin and MAP-2) were prepared for these studies as described in chapter 2 of this report. MAP-2 was trace phosphorylated by the catalytic subunit of protein kinase A (Sigma). Typically, 500 units of kinase were reconstituted in 25 µl of DTT (50 mg/ml) at room temperature for 10 minutes and incubated immediately with 1.5 mCi [γ-^{32}P]ATP, 20 µM unlabeled ATP, and assembly buffer at 37°C for 30 minutes in the presence of approximately 40 mg heat-treated MAPs. Unincorporated ATP was removed by gel filtration on a BioGel A 1.5-m column during separation of MAP-2 from tau proteins as described (see chapter 2).

Proteolytic cleavage of MAP-2. The 11 kD form of recombinant HIV proteinase (Graves *et al*., 1988) was used in these investigations and generously provided by Dr. Ben M. Dunn, of the Department of

Biochemistry and Molecular Biology, University of Florida. All digestions of MAP-2 (5 μM) with this protease were performed at 37°C in enzyme buffer (50 mM Mes, 2 mM EDTA and 1 M NaCl pH 6.2) upto 100 minutes. Concentrated stock solutions of the proteinase (8 mg/ml) were first serially diluted with 50 mM sodium acetate (pH 4.7) containing 2.5 mM EDTA, 1 mM DTT, 500 mM NaCl and 10% (v/v) glycerol and then diluted to the desired final concentration. The protease was heat inactivated at 100°C for 10 minutes and the sample was dialyzed at 4°C for 5 hours against assembly buffer to reduce salt levels prior to further analysis. Some samples were incubated with thrombin (8 units/ml) at 37°C for 30 minutes in assembly buffer to release the MT-binding region.

Radiolabeled purified protein was treated with HIV proteinase (8 μg/ml) as described to resolve potential differential cleavage patterns in MAP-2a and -2b. Samples were run on a 4% SDS-polyacrylamide gel containing 2 M urea as described by Bloom *et al.* (1985). Bands were visualized by autoradiography and quantitated using a BioImage densitometer with equal counts loaded per lane.

Analysis of polypeptide fragments. In sedimentation experiments proteolytically cleaved MAP-2 samples were incubated with taxol-stabilized microtubules to separate MT-unbound and bound fractions using the binding assay described in chapter 2. Polypeptide fragments were electrophoresed on a 7.5% SDS-polyacrylamide gel (Laemmli, 1970) and visualized by Coomassie blue staining, silver staining or autoradiography. In competition assays, trace-phosphorylated intact MAP-2 or proteolytically produced MAP fragments were incubated with taxol-stabilized MTs in the presence or absence of 2.0 mM m_2' peptide

(VTSKCGSLKNIRHRPGGGRVK) for 30 minutes at 37°C before centrifugation. The m_2' peptide was synthesized by Jon Pol, Microchemical Synthesis Core, Emory University. This peptide is known to displace MAP-2 from the microtubule lattice (Joly and Purich, 1990).

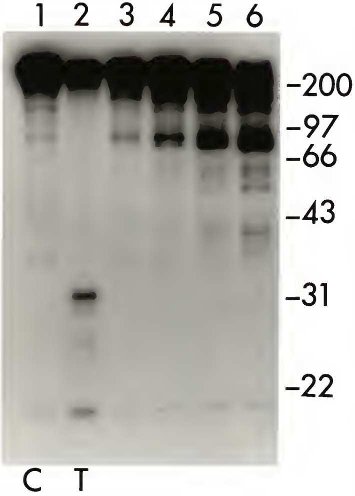
The assembly assays were performed under two conditions. First, microtubule proteins (2.0 mg/ml) in assembly buffer were incubated with 1 mM GTP in the presence or absence of the HIV proteinase (8 and 80 μg/ml) and light-scattering was monitored using a Varian 210 spectrophotometer at 30°C (see chapter 2). After reaching a steady-state (approximately 30 minutes), a indication of complete polymer mass formation, samples were centrifuged at 160,000 x g in a Beckman Airfuge at 30°C for 20 minutes. Supernatant and pellet fractions were run on a 7.5% reducing gel and visualized by silver stain.

In a second set of assembly experiments, purified MAP-2 (5 μM) was treated with or without 80 μg/ml of proteinase, desalted and the treated protein (1.5 μM) was added to 1.2 mg/ml tubulin. The tubulin was pre-incubated at 37°C for 5 minutes in the presence of a GTP-regenerating system (2 units/ml acetate kinase, 10 mM acetyl phosphate and 1 mM GTP) in assembly buffer. The change of absorbance was then monitored at 350 nm at 30°C for 30 minutes.

Results

Wallin *et al.* (1990) observed that several microtubule-associated proteins are cleaved by HIV and avian myeloblastosis virus proteases. I was motivated to examine the action of HIV proteinase directly on MAP-2. Lane 1 in Figure A-1 represents MAP-2 without the HIV enzyme, and for

Fig. A-1: Characterization by gel electrophoresis of MAP-2 digestion with the HIV proteinase. A sample of bovine-brain heat-treated MAP-2 (1.0 mg/ml) in 50 mM Mes, 2 mM EDTA and 1 M NaCl (pH 6.2) was incubated with 5.0 µg/ml HIV proteinase at 37°C for 3, 10, 30 and 100 minutes (lanes 3-6, respectively). Lane 1 contains undigested MAP-2. Lane 2 shows MAP-2 in assembly buffer after digestion with 8 units/ml thrombin for 30 minutes. C, control; T, thrombin.


1
2
3
4
5
6
–200
–97
–66
–43
–31
–22
C
T

comparison lane 2 shows how thrombin cleaves the protein into two fragments. As shown in lanes 3 to 6 of Figure A-1, I found that the exposure of MAP-2 to recombinant HIV proteinase at a 10:1 molar ratio resulted in extensive MAP-2 degradation. The 12% SDS gels used in these experiments tend to compress the high-molecular weight species, and the breakdown of MAP-2 was most evident in terms of the time-dependent accumulation of a prominent M_r 72,000 species and at least five others less abundant bands, including one with a M_r value of 130,000-140,000. For these experiments [γ-^{32}P]-labeled MAP-2 was used, but companion SDS-polyacrylamide gels were silver stained to verify that a similar pattern of breakdown products was observed (for example, see lane 1 of Fig. A-6).

Since adult MAP-2 occurs as two closely migrating bands (designated MAP-2a and MAP-2b), I considered the possibility that the HIV proteinase might selectively cleave one of these MAP-2 species. Accordingly, I used 4% SDS gels containing 2 M urea (Bloom *et al.*, 1985) to obtain better resolution of these proteins, and the relative abundance of MAP-2a and -2b was analyzed by densitometry of autoradiograms (see Fig. A-2). I obtained virtually identical results in three separate experiments, and these data indicate that there is little or no preference in HIV action on these high-molecular weight species.

To learn whether the fragmentation pattern for MAP-2 might change in an enzyme concentration-dependent manner, I next worked at 0.5, 1.5, and 5 µg/ml of the HIV proteinase. The data shown in Figure A-3 correspond to the time-course of cleavage over a 100 minutes interval

Fig. A-2: Analysis of MAP-2a and -2b susceptibility upon HIV protease treatment. MAP-2 (pH 6.2) was digested with 2.5 μg/ml of protease as described, electrophoresed and visualized by autoradiography. MAP-2a (☐) and MAP-2b (■) were quantitated by densitometry.

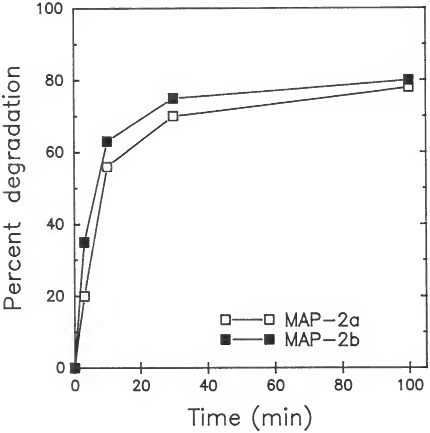
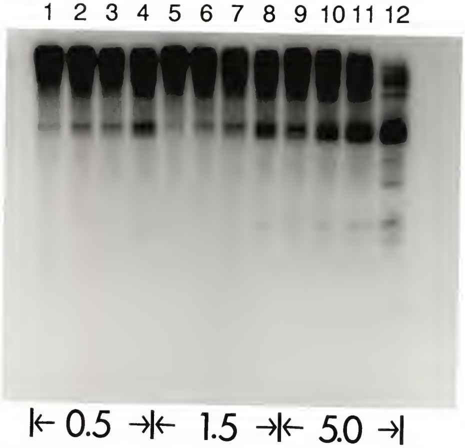

Percent degradation
100
80
60
40
20
0
0
20
40
60
80
100
Time (min)
□—□ MAP-2a
■—■ MAP-2b

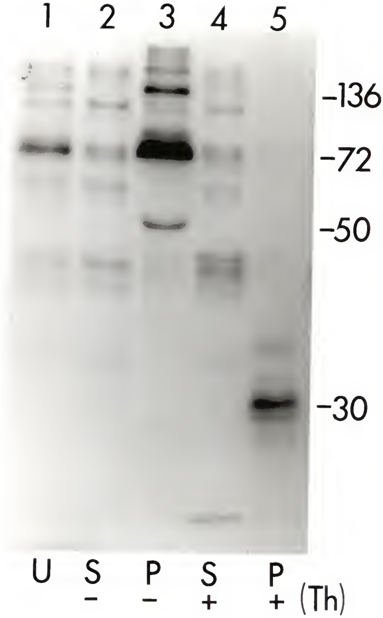
Fig. A-3: Digestion pattern of MAP-2 with various enzyme concentrations. Purified MAP-2 (1.0 mg/ml) was incubated with HIV protease at 0.5 (lanes 1-4), 1.5 (lane 5-8) and 5.0 (lane 9-12) µg/ml for 3 (lanes 1,5 and 9), 10 (lanes 2, 6, and 10), 30 (lanes 3, 7 and 11) and 100 (lanes 4, 8 and 12) minutes. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.


1 2 3 4 5 6 7 8 9 10 11 12
|← 0.5 →|← 1.5 →|← 5.0 →|

following enzyme addition. Again, I found that the M_r 72,000 fragment was most abundant, and I found that there were two closely migrating species in this narrow molecular weight range. I also observed several more faintly stained species that accumulated along with the M_r 72,000 species. Several of these lower molecular weight species (M_r 27,000 - 60,000) are evident in lanes 10-12 of Figure A-3. I conducted thrombin cleavage experiments subsequent to HIV proteinase action to identify those proteolytic fragments that contained the MT-binding region. The results of such an experiment are shown in Figure A-4. Lane 1 depicts the HIV protease fragmentation pattern for MAP-2, and lanes 2 and 3 correspond to the supernatant and pellet fractions obtained after incubating HIV proteinase-cleaved MAP-2 with taxol-stabilized microtubules, followed by ultracentrifugation to separate MT-unbound protein in the supernatant (S) fraction from MT-bound protein in the pellet (P) fraction. MAP-2 binds reversibly to microtubules and the supernatant fluid always contained some MAP-2, although at a lower level relative to MT-bound MAP-2. Nonetheless, at least three bands (M_r 136,000, 72,000 and 50,000) exhibited preferential binding to microtubules. Upon treatment with thrombin, these and other nearby minor polypeptides no longer were observed in the pellet fraction (Lane 5) which largely contained the C-terminal MT-binding region of MAP-2 as formed by thrombin action (see Lane 2 of Fig. A-1 for comparison). These data suggest that the M_r 136,000, 72,000 and 50,000 bands contain the MT-binding domain and include the C-terminal end of MAP-2.

I conducted a related experiment to test whether the M_r 72,000 species contains the microtubule-binding domain, using a peptide

Fig. A-4: Determination of HIV protease-generated microtubule-binding fragment. MAP-2 (pH 6.2) was incubated with 5 µg/ml of HIV protease for 30 minutes at 37°C (lane 1). The digested sample was then dialyzed and incubated in the absence (lanes 2 and 3) or presence (lanes 4 and 5) of 8 units/ml thrombin for 30 minutes at 37°C. All samples were then incubated with taxol-stabilized microtubules and centrifuged [*see chapter 2*]. Lanes 2 and 4 represent supernatant (S) fractions and lanes 3 and 5 represent pellet (P) fractions. U, untreated.


1 2 3 4 5
-136
-72
-50
-30
U S P S P
− − + + (Th)

analogue corresponding to the second repeated peptide sequence in the MAP-2 microtubule-binding region. Joly and Purich (1990) demonstrated that this peptide competitively displaces intact MAP-2 from MAP-containing microtubules. As shown in Figure A-5, the m_2' peptide prevented binding of both intact MAP-2 (lanes 1-4) and the M_r 72,000 and 50,000 polypeptides (lanes 5-8) to microtubules. This finding indicated that the presence of the M_r 72,000 species in the MT pellet fraction depends on binding to microtubules. This is consistent with the conclusion that M_r 72,000 species contains the C-terminal MT-binding region of MAP-2.

Wallin *et al.* (1990) reported that HIV proteinase treatment of microtubule protein resulted in loss of reassembly properties. They attributed this finding to cleavage of microtubule-associated proteins. Their observation and conclusion that HIV proteinase altered the ability of MAP-2 to promote tubule assembly was confusing, since my experiments indicated that HIV proteinase action did not alter the microtubule-binding domain of MAP-2. To investigate this issue further, I compared my conditions using purified MAP-2 with their studies with recycled microtubule protein (*i.e.*, samples containing α and β tubulin, MAP-2, and tau proteins). As shown in Figure A-6, HIV proteinase readily cleaves isolated MAP-2 (lanes 1 and 2), but MAP-2 in the unfractionated microtubule protein seemed less susceptible to cleavage (note MAP-2 bands still remaining lanes 4 and 6). In companion experiments, I also found that HIV treatment under their buffer conditions at pH 6.8 in the absence of salt had little or no effect on microtubule assembly.

Fig. A-5: Competition assay of proteolytically cleaved MAP-2 by synthetic m_2' peptide. Intact MAP-2 in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of m_2' was incubated with taxol-stabilized microtubules. MT-unbound (S) and bound (P) fractions were separated by centrifugation. MAP-2 cleaved by HIV protease (HIV enz) action, was similarly incubated in the absence (lanes 5 and 6) or presence (lanes 7 and 8) of peptide as previously described. Lanes 1, 3, 5 and 7 represent supernatants; lanes 2, 4, 6 and 8 represent pellet fractions.

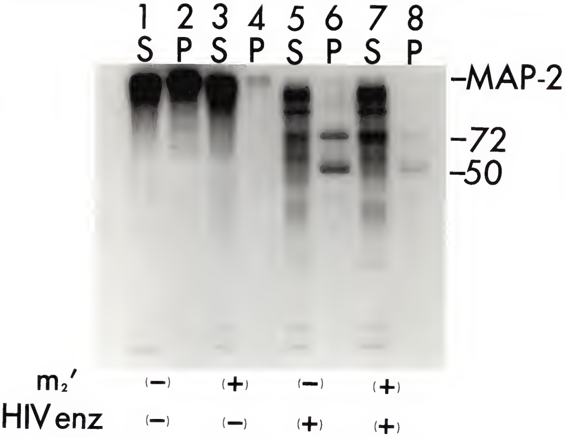
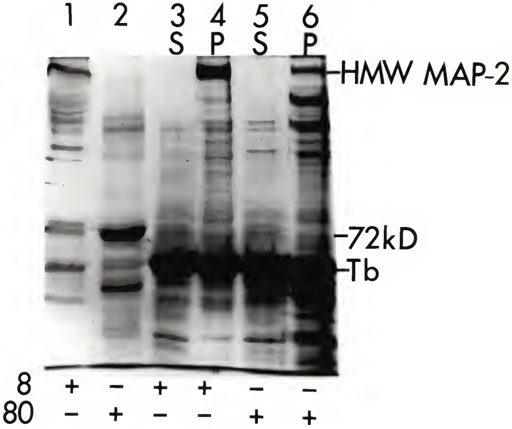

1 S
2 P
3 S
4 P
5 S
6 P
7 S
8 P
–MAP-2
–72
–50
m₂′ (–) (+) (–) (+)
HIV enz (–) (–) (+) (+)

Fig A-6: Analysis of microtubule protein assemblies following HIV protease treatment. Microtubule proteins were incubated in assembly buffer with 1 mM GTP in the presence of either 8 (lanes 3 and 4) or 80 (lanes 5 and 6) µg/ml of protease [*see Methods*]. Lanes 1 and 2 show digestion patterns of MAP-2 (pH 6.2) upon incubation with 8 and 80 µg/ml of HIV protease respectively. Assembled microtubule proteins were centrifuged as described, electrophoresed on a 7.5% denaturing gel and visualized by silver stain. Lanes 3 and 5 represent supernatant (S) and lanes 4 and 6 represent pellets (P). The bands for high molecular weight (HMW) MAP-2, the 72 kD fragment and tubulin (Tb) are indicated.

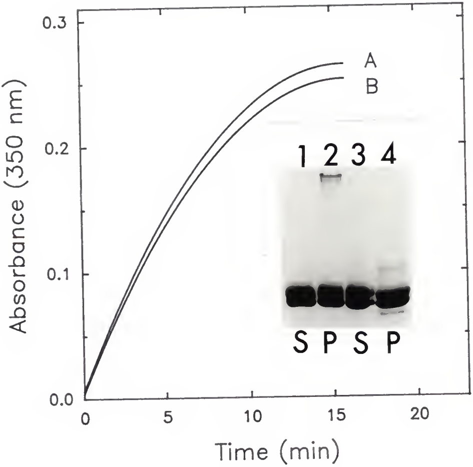

1
2
3
S
4
P
5
S
6
P
HMW MAP-2
72kD
Tb
8 + − + + − −
80 − + − − + +

Accordingly, I assessed microtubule polymerization properties with isolated MAP-2 under conditions that I found to be most favorable for cleavage. I worked at pH 6.2 in 1 M NaCl, favorable for the HIV proteinase cleavage, followed by desalting of MAP-2 fractions by dialysis. When isolated MAP-2 was incubated in the absence or presence of 80 μg/ml HIV proteinase, desalted, and then recombined with tubulin (10 mg/ml), both samples exhibited the capacity to polymerize (Fig. A-7). The inset to this figure clearly shows that the M_r 72,000 species was generated under these conditions by HIV proteinase action. Altogether, these experiments were consistent with the conclusion that HIV proteinase produces a M_r 72,000 fragment that contains the MT-binding region. Such cleavage does not significantly alter the assembly-promoting properties of MAP-2.

Discussion

The life cycle of retroviruses involves the action of virally-encoded proteinases to cleave single polypeptide precursors. These proteins are initially formed as a single translation product from the viral mRNA template, and constituent structural proteins and processing enzymes are formed by proteolysis. The polypeptide proteinase is part of the original protein and accumulates in host cells at stoichiometric levels compared with the other viral proteins and enzymes. Such accumulation may lead to proteolytic action on other nearby non-viral proteins present in host cells. In view of my interest in MAP-2 structure, I was motivated to study the action of recombinant

Fig. A-7: Analysis of microtubule assembly stimulated by MAP-2. Heat-treated MAP-2 was incubated in the absence (curve A) or presence (curve B) of 80 µg/ml of HIV protease and treated as described. MAP-2 was subsequently incubated with 1.2 mg/ml tubulin in a regenerating system at 30°C. The change of absorbance *versus* time was measured as a direct measurement of microtubule polymerization. Inset: Both samples were then centrifuged to separate microtubule-bound and unbound, intact and digested, MAP-2. Lanes 1 and 2 represent sample A and lanes 3 and 4 represent sample B. Lanes 1 and 3 are supernatants; lanes 2 and 4 are pelleted fractions.


0.3
0.2
0.1
0.0
Absorbance (350 nm)
0
5
10
15
20
Time (min)
A
B
1 2 3 4
S P S P

HIV proteinase on this high-molecular weight cytomatrix protein. During this work, Shoeman *et al.* (1990) reported that HIV-1 proteinase cleaves the intermediate filament proteins vimentin, desmin, and glial fibrillary acidic protein. Wallin *et al.* (1990) also reported on the proteolytic cleavage of microtubule-associated proteins by retroviral proteinases, and they concluded that such degradation of a mixture of bovine brain MAPs was attended by the loss of their ability to stimulate microtubule assembly. However, a 72(73) kD fragment generated upon treatment was shown to bind taxol-stabilized microtubules. My findings extend their work by identifying the proteolytic cleavage pattern of HIV proteinase using isolated MAP-2, but I have not observed any significant changes in tubulin polymerization behavior in the presence of HIV proteinase-cleaved MAP-2.

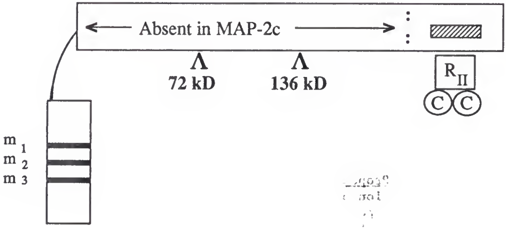
Recently, Johnson *et al.* (1991) studied the *in vitro* degradation of MAP-2 and spectrin by the calcium-dependent neutral proteinase calpain. They made the very interesting observation that sensitivity to calpain-induced hydrolysis was markedly different for so-called recycled MAP-2 (*i.e.*, that obtained from assembly/disassembly purification of microtubules) *versus* that obtained from total brain heat-stable MAP-2. One explanation for this behavior is that recycled MAP-2 is selectively enriched by microtubule-binding, and the results of Jameson and Caplow (1981) indicate that phosphorylated MAP-2 binds more weakly to microtubules. Indeed, Tsuyama *et al.* (1987) found that total brain MAP-2 was highly phosphorylated, and this suggests that the results of Johnson *et al.* (1991) may be related to the extent of MAP-2 phosphorylation. Indeed, most recently Johnson *et al*. (1993) found that protein kinase A inhibits calpain-action on MAP-2.

The results of cDNA sequencing experiments suggest that the structural organization of MAP-2 is quite similar for the mouse, rat, and human proteins (Kosik *et al*., 1988; Wang *et al*., 1988; Kindler *et al*., 1990). Likewise, observations in the laboratory confirm that the last three hundred amino acid residues in the C-terminal microtubule-binding region of bovine brain MAP-2 are virtually identical with the mouse sequence (R. L. Coffey *et al*., submitted). The most prominent structural features of MAP-2 are shown schematically in Figure A-8. Adult MAP-2ab corresponds to the full-length molecule whereas embryonic MAP-2 lacks the 1,342 amino acid portion that connects the tubule-binding and protein kinase regulatory subunit-binding domains that are common to both isoforms (Rubino *et al*., 1989; Obar *et al*., 1989). In this respect, my observation that the HIV proteinase leads to the formation of M_r 72,000 and M_r 136,000 species containing the C-terminal MT-binding domain indicates that the proteinase cleaves within the 1,342 amino acid region. This suggests that such cleavage is only possible in the adult form.

The sequence of bovine MAP-2 has not been fully defined; so the derived amino acid sequences for mouse, rat, and human MAP-2 were inspected in attempts to identify likely HIV cleavage sites. The consensus cleavage sequences were used for HIV-1 proteinase action on various viral polypeptide (Skalka, 1989). Sequences were scanned for potential cleavage sites made up either of an aromatic amino acid

Fig. A-8: Proposed sites of HIV protease action on MAP-2. A cartoon representation of MAP-2 with the projection arm and MT-binding region shown. The open triangles indicate the approximate sites of HIV protease cleavage in the projection arm domain based on MAP-2 fragmentation patterns (*see Fig. 1 and 6*).

Localization of HIV protease cleavage sites on MAP-2



followed by proline or two adjacent hydrophobic amino acids. While all three MAP-2 sequences contained numerous (~ 15 or more) potential cleavage sites, each lacked other flanking residues that are characteristically found in good substrates for human immunodeficiency virus proteinases. This does not exclude the possibility that any of these sites can serve as a substrate for these enzymes, but the lack of such flanking sequences suggests that cleavage, if it does occur, is not characterized by high-affinity enzyme-substrate interactions.

Despite several attempts, I was unable to replicate the results of assembly experiments by Wallin *et al.* (1990). They observed that HIV protease action on microtubule protein (tau, tubulin and MAPs) resulted in significant inhibition of microtubule assembly. In the experiments with either microtubule protein or isolated MAP-2, proteolysis was clearly observed (see Fig. A-7 inset) thus, my inability to confirm their observation of inhibition is probably unrelated to the failure of the proteinase to cleave. Finally, while HIV proteinase cleavage does not inhibit microtubule polymerization, other aspects of cytoskeletal organization might result from such proteolysis.

Hirokawa (1988) has suggested that MAP-2 plays a role in cross-linking dendritic microtubules, and my findings suggest that such cross-links could be lost upon HIV proteinase treatment. Likewise, as illustrated in Figure A-8, MAP-2 contains a high-affinity binding site for the regulatory subunit of 3′,5′-cAMP stimulated protein kinase (Vallee, 1986). Although the cellular role of this regulatory subunit binding to the cytoskeleton is uncertain, the site is located very near the N-terminus of MAP-2 and HIV proteinase cleavage would remove this site entirely from the M_r 72,000, 136,000 and 50,000 species that should remain bound to microtubules. The R_{II} dimer displays an internal rotational diad axis of symmetry (Taylor *et al.*, 1990), and there remains the possibility that pairs of MAP-2 molecules can bind to a shared R_{II} dimer, thereby creating a MAP-2:R_{II}:MAP-2 complex capable of cross-linking microtubules. If the high-affinity MAP-2 binding sites on R_{II} play this cross-bridging role, then HIV proteinase or some related viral proteinase may disturb cytoskeletal organization by severing these inter-microtubule links. It also should be noted that Flynn and Purich (1987) found that the M_r 28,000 microtubule-binding fragment produced by thrombin binds to neurofilament proteins and microtubules, suggesting that HIV proteinase action would not be expected to disrupt MAP-2 interactions with neurofilaments. Nonetheless, the consequences of retroviral infection of neurons remains an uncharted area in terms of changes in cytoskeletal components, and the experiments described by Wallin *et al.* (1990) and myself may serve to encourage further research concerning cytoskeletal involvement in virally induced neuropathy.

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BIOGRAPHICAL SKETCH

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